THE UNIVERSITY OF HULL

Studies In Iron Metabolism In *Mycobacterium smegmatis, Mycobacterium avium* and other Mycobacteria

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by

Kevin Alexander McCready, B.Sc.

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NO INFORMATION IS MISSING

Gold is for the mistress, silver for the maid,
Copper for the craftsman cunning at his trade,
"But", said the baron, sitting in his hall,
"Iron, cold iron, is the master of them all".

R. Kipling. "Cold Iron"

To my parents

and my wife.

Acknowledgements

I would like to thank all those people, too numerous to mention, who made my period of study at Hull University such a happy and rewarding experience. My thanks especially go to Professor E. A. Dawes for allowing me to work in his department, and to my supervisor Dr. C. Ratledge for his guidance and friendship during my three years as his Ph.D. student. I also owe a debt of gratitude to both Dr. P. Botham for his friendship and help, and to Dr. M. C. Stepehenson for her encouragement and advice. I would also like to thank Dr. D. Ewing for his help with the n.m.r. spectroscopy, Mr. A. Roberts for performing the mass spectrometry, Mrs. E. Eagle for her technical assistance, and Mrs. B. M. Wilson for typing the manuscript.

Some information contained in this thesis has also been presented in scientific communications, listed below.

Ratledge, C and McCready, K. A. (1977) *Int. J. Syst. Bact.* 27, 288.

McCready, K. A. and Ratledge, C. (1978) *Biochem Soc. Trans.* 6, 421.

McCready, K. A. and Ratledge, C. (1979) *J. Gen. Microbiol* 113, 67.

Abstract

Research in this thesis is concerned with iron metabolism in *Mycobacteria,* with special regard to *Mycobacterium smegmatis*. A study of the enzyme ferrimycobactin reductase from this organism is made. This enzyme is believed to be responsible for the release of iron from ferrimycobactin at the cell wall-cytoplasm interface. The aim of this study is to characterise the activity further, with a view to understanding its mode of action.

Additionally, an examination is carried out of the levels of certain important cellular components during iron-deficient and iron-sufficient growth of *M. smegmatis*. This is to observe what 'economies' the cell must make in times of shortage of an essential nutrient such as iron. The response of the cell, when growing iron-deficiently, is monitored when a sudden supply of iron is presented to the cell. The iron is added in several different 'forms' to determine whether these forms of addition of iron influence the response of the cell.

Mycobactin is also employed in an attempt to resolve the overlap between *M. avium* and *M. intracellulare*. By examination of the mycobactins produced by strains of these organisms, it is hoped to determined whether the *M. avium-intracellulare* complex consists of one or two species.

Lastly, a brief study is made of several *Mycobacteria* that are dependent upon mycobactin for growth, to determine whether these organisms produce a mycobactin of their own.

Contents

INTRODUCTION

I Iron and Its Availability in Nature

The entire planet Earth is approximately one-third iron, the concentration of which is higher than any other element. In the Earth's crust iron is the fourth most abundant element, the only metal more abundant being aluminium (Zajic, 1969). However iron is too reactive to be found free and is rapidly oxidised under aerobic conditions. Its chief ore is haematite (Fe_2O_3), but it is also found as magnetite (Fe_3O_4), limonite ($2Fe_2O_3.3H_2O$) and pyrites (Fe_2S). Concentration of iron in the soil is roughly 3.8%, but this varies with the type of soil; the Earth's oceans however, only contain 0.01 p.p.m. iron, the concentration increasing with depth (Bowen, 1966). The concentration in river water is higher, varying from 0.01 to 0.4 p.p.m. (Bowen, 1966).

Iron in the reduced state is soluble and this element, along with most others, was present in this form on the surface of primitive Earth (Ponnamperuma, 1971; Neilands, 1973). Presumably due to the versatility of this element (see Section II) iron became established as an important component of the biological systems of primitive micro-organisms. But with the development of an oxygen-containing atmosphere, due to the evolution of oxygen-evolving blue-green algae and eventually plants, iron became available only in the oxidised state, which of course is insoluble (Neilands, 1972, 1973).

Unchelated iron in aqueous solution at physiological pH exists as an insoluble hydroxide, the solubility product constant for which has been variously estimated at $1.1X\ 10^{-36}$ (Kolthoff and Elving, 1962) and $10^{-38.7}$ (Lankford, 1973). The solubility of iron is also dependent upon pH, as reflected by the solubility equilibrium of $Fe(OH)_3$, where the solubility of Fe^{3+} is inversely proportional to $[OH^-]^3$. Thus, at pH7.0, the solubility of Fe^{3+} is in the order of 10^{-15} M.

Thus, although an abundant element, this physiological barrier to its availability has necessitated micro-organisms devising means to solubilise and transport iron. This has been mainly achieved by the synthesis of specific iron-chelating compounds, the properties of which are discussed later (see Section VI and VII).

II Importance of Iron In Nature

Iron owes its importance as an essential element in biological systems to the versatility that it displays. It has in fact been dubbed the most versatile of all biochemically active metal ions (Eichorn, 1964). Iron possesses two stable valencies and a wide range of oxidation-reduction potentials for the Fe^{2+}/Fe^{3+} couple; potentials between +300mV in certain a-type cytochromes and -500mV in some iron-sulphur proteins have been recorded (Neilands, 1974). In addition, iron in both the ferrous and ferric states, tends to form chelates readily with given ligands. This is due to the relatively small size of the ions, coupled with a high charge and a number of d-orbitals available for bonding. Iron in the oxidised form displays a distinct preference for oxygen ligands e.g. as in hydroxamates, while

ferrous ions bind preferentially to all nitrogen ligands, such as the phenanthrolines (Phillips and Williams, 1966).

Iron is an essential component of a wide range of proteins in microbial systems, which may be conveniently divided into four categories (Coughlan, 1971):-

(i) The haem enzymes, which include the cytochromes, whose function in electron transport is well documented, and catalase and peroxidase, which function to break down peroxide molecules.

(ii) The oxygenases, which catalyse the incorporation of oxygen into their substrates e.g. tryptophan dioxygenase.

(iii) Ferroflavoproteins, which contain iron and riboflavin (as FMN or FAD) as prosthetic groups e.g. xanthine oxidase, which are usually involved in dehydrogenation reactions.

(iv) Low molecular weight electron transfer proteins e.g. ferridoxin, which are found widely in microbial systems where electron transfer occurs e.g. photosynthesis, N_2-fixation, oxidative phosphorylation.

The importance of iron is also highlighted by the fact that, with the possible exception of the lactic acid bacteria, iron is present in all living systems. *Lactobacilli* have possibly evolved in special environments where iron was absent, but all other nutritional requirements were met e.g. the oral cavity (Neilands, 1973).

III Iron and Pathogenicity

Iron, as has been shown, is an essential nutrient for bacterial growth, and it has recently become apparent that the ability to acquire iron by a micro-organism from the host

is an important feature of pathogenicity. However, as early as 1944, Schade and Caroline (1944, 1946) observed that the iron-binding proteins in egg-white and serum were involved in the inhibition of growth of *Shigella dysenteriae*.

Although large quantities of iron are present in host tissues, it is in a bound form, usually as ferritin, or bound to the serum protein, transferrin. Both transferrin and ferritin are normally only 20-30% saturated with iron and have extremely high association constants of approximately 10^{36} for iron. The amount of free iron in such cases has been calculated as roughly $10^{-18}\underline{M}$, which is a factor of several thousand fold too small to support bacterial growth (Bullen *et al*, 1972). However, if the iron-binding capacity of the protein is saturated with iron, or iron-containing compounds are injected into the host animal, resistance to infection is lowered considerably (Bullen *et al*, 1972).

Hence, in order to grow *in vivo*, pathogenic bacteria must be capable of acquiring iron from one of the sources of bound iron. This is believed to be achieved by means of the specific iron-chelating agents produced by bacteria, whose affinity for iron is at least as great as that of the iron-binding proteins (Weinberg, 1971; Bullen *et al*, 1974; Weinberg, 1978).

The growth of virulent mammalian tubercle bacilli ($H37R_V$) and the attenuated BCG bacillus are inhibited by many mammalian sera (Kochan *et al*, 1963; Kochan *et al*, 1969). The degree of saturation of transferrin inversely affects the bacteriostatic effect of the serum, which can be completely abolished by the addition of iron to saturate its iron-binding capacity (Kochan, 1969). However, both

mycobactin and exochelin (the intracellular and extracellular iron-chelating agents produced by *Mycobacteria*) are capable of reversing the growth inhibitory effects of serum (Kochan *et al*, 1971; Macham *et al*, 1975, 1977).

Enterochelin, the extracellular iron-chelating agent produced by *Enterobacteriaceae*, is also capable of removing iron from the iron-transferrin complex (Bullen *et al*, 1972). Results similar to those obtained with the above organisms have been recorded using a wide range of other bacteria (Bullen *et al*,1974). However direct *in vivo* evidence for the involvement of these iron-chelating compounds has yet to be obtained, and further investigations are required before their mode of action are fully understood. In addition, although iron acquisition is important with regards to pathogenicity, other factors are presumably involved, as many organisms that can successfully acquire iron from their host are not pathogenic e.g. *M. smegmatis*.

IV Effects of Iron-Deficiency On Micro-Organisms

The effects of iron-deficiency on micro-organisms are discussed below in terms of haem iron and non-haem iron-containing material, as well as compounds affected that do not contain any iron. That a deficiency of iron should exert an effect on cell function is apparent from the importance of iron in microbial systems, but the way in which micro-organisms respond to iron deficiency is indicative of the importance of the various iron-containing compounds in the cell. During iron-deficient growth, is the concentration of one "class" of iron-containing compound e.g. haem iron, maintained at the expense of the other; or are the concentrations of certain essential compounds from both classes

of iron-containing compounds maintained during iron-deficient growth?

The response of micro-organisms to iron-deficiency enables one to see the cell's ability to cope with a situation that must occur frequently *in vivo*. Koch (1971) has defined the existence of micro-organisms *in vivo* as one of "feast and famine". He argued that, as opposed to the *in vitro* environment where the concentrations of nutrients are strictly controlled, the nutrient concentrations in the *in vivo* environment will fluctuate between extremes of scarcity and plenty. Examination of the effects of iron-deficiency *in vitro* will thus afford an insight into the possible concentrations of iron-containing compounds in micro-organisms when growing in their natural environments.

(a) Haem iron-containing Compounds. In aerobic organisms, the total cytochrome concentration falls during iron-deficient growth (Van Heyningen, 1955; Webley *et al*, 1962). *Candida utilis* displays a 3-4 fold difference in cytochrome concentrations between iron-deficient and iron-sufficient cells (Clegg and Garland, 1971; Light, 1972), while Grossman *et al*, (1974) observed that cytochrome b was the most affected cytochrome during iron-deficient growth of this organism. Bound cytochrome b was absent in *Rhodopseudomonas spheroides* (Agaldis *et al*, 1974), and in *Escherichia coli* and *Pseudomonas flourescens*, cytochrome b was the most affected by iron-deficiency being 3-4 fold lower during iron-deficient growth (Rannie and Bragg, 1973; Kiprianova and Kornushenko, 1969).

Other haem iron-containing proteins e.g.catalase and peroxidase, decline in activity during iron-deficient growth of a wide range of bacteria and fungi (Pappenheimer and Hendee 1947; Healey *et al*, 1955; Webley *et al*, 1962). Peroxidase from *Candida guilliermondii* was inactive during iron-deficient growth, while catalase activity was only 1.25% of that during iron-sufficient growth of this organism (Kauppinen, 1968). These enzymes were present at only 15% of the activity found in iron-sufficient cells, during iron deficient growth of *M. smegmatis* (Winder and O'Hara, 1964). The effects of iron-deficiency on the enzymes of haem and porphyin biosynthesis are discussed separately in this thesis (Section V).

(b) Non Haem Iron-Containing Compounds. Non haem iron-containing enzymes e.g succinate dehydrogenase, NADH dehydrogenase, are also affected by iron-deficiency. *C. utilis,* during iron-deficient growth, completely lacks one type of NADH dehydrogenase (Grossman *et al,* 1974), while Clegg (1972) observed that the activity and composition of this enzyme were altered during iron-deficient growth of this organism. Similar results have been obtained using *Azotobacter vinelandii* and *Micrococcus denitrficans* (Der Vartanian, 1972; Imai *et al*, 1968), while NADH dehydrogenase activity in iron-deficient *M. smegmatis* was 75% of that observed during iron-sufficient growth (Winder and O'Hara, 1964). Succinate dehydrogenase of iron-deficient *Neurospora crassa* was only 20% of that observed during iron-sufficient growth (Healey *et al,* 1955) while this enzyme was similarly affected in iron-deficient *C. guilliermondii* (Kauppinen, 1968). The soluble

ferredoxin of *Cl. pasteurianum* is not synthesised under iron-deficient conditions but, instead, a metal-free flavoprotein (Flavodoxin) is synthesised which performs the same functions as ferredoxin (Knight and Hardy, 1966). Although there are not many examples quoted in the literature, it would not be surprising for micro-organisms to synthesise novel compounds e.g. flavodoxin or structurally altered compounds e.g. NADH dehydrogenase of *C. utilis* etc. during iron-deficient growth. Thus the available iron would be used for the synthesis of essential or unreplacable compounds eg. cytochromes, thereby increasing the micro-organism's potential for survival during stringent growth conditions.

Total non-haem iron declines under iron-deficient conditions. In *C. utilis*, non-haem iron concentration was reduced thirty fold during iron-deficient growth (Light, 1972) while similar observations were made with *E. coli* (Rannie and Bragg, 1973) and *Corynebacterium diphtheriae* (Righelato, 1969). Due to the role played by non-haem iron in electron transport, iron-limitation may affect energy-yielding processes of micro-organisms. *C. utilis* during iron-deficiency, preferentially uses acetyl-coenzyme A for ethyl acetate formation, rather than being oxidised via the TCA cycle, which occurs under iron-sufficient growth conditions (Thomas and Dawson, 1978). A loss in energy-conservation in the mitochondia of iron-deficient *C. utilis* has been observed by Light and Garland (1971), occurring at the site 1 segment of the respiratory chain. This was supported by the differences in inhibitor sensitivity between iron-deficient and iron-sufficient

C. utilis when oxidising NAD-linked substrates (Light *et al*, 1968; Ohnishi and Schleyer, 1969). This implies the presence of an iron-dependent component at site 1 of the respiratory chain i.e. between NADH and cytochrome c. Similar results were obtained with *E. coli*, where energy-coupling mechanisms were affected by iron-deficiency (Rannie and Bragg, 1973). The effects of iron-deficiency on aerobic energy-yielding processes have been summarised by Lankford (1973) who stated that micro-organisms tend to change to anaerobic glycolysis to meet their energy requirements, on the suppresion of aerobic glycolysis by iron-deficiency.

(c) Other compounds. Other enzymes, that do not contain iron are also affected by iron-deficient growth of micro-organisms. Aconitase activity in iron-deficient *C. guilliermondii* decreases with iron concentration, to 10% of the activity seen during iron-sufficient growth, when iron is present at 0.01µg/ml (Kauppinen, 1963). In contrast, aldolase activity in this organism increases during iron-deficient growth, the activity increasing four fold on lowering the iron concentration from 0.5µg/ml to 0.01µg/ml (Kauppinen, 1963). Alcohol dehydrogenase activity in this organism also increased with decreasing iron concentration, increasing four fold over the iron concentration range shown above (Kauppinen, 1968). Glycerol dehydrogenase also declines in activity in iron-deficient *M. smegmatis*, activity being 3-4 fold lower in iron-deficient compared to iron-sufficient cells (Winder and O'Hara, 1964).

Certain compounds may be produced maximally under iron-deficient conditions (other than the phenolates and hydroxamates which are discussed in Sections VI and VII).

Riboflavin is excreted by both *Candida* species (Tanner *et al*, 1945) and *Cl. acetobutylicum* (Yamasaki, 1941) during iron-deficient growth. However the reason for this effect is not yet understood (Light and Clegg, 1974). Toxin production is promoted during iron-deficient growth of some bacteria eg. *C. diphtheriae, S. shigea* and various *Clostridia* species (Sickless and O'Leary, 1968; Righelato, 1969; Murrata *et al*, 1956; Latham *et al*, 1962). The toxin contains protein normally contained in cytochrome c, and it is cytochrome c instead of toxin that is maximally produced during iron-sufficient growth (Sickless and O'Leary, 1968).

The morphology of iron-deficient organisms is also altered. Iron-deficient *M. smegmatis* increases several-fold in length, (Winder and O'Hara, 1962) while similar observations have been made with iron-deficient *E. coli* (Ratledge and Winder, 1964) and *C. utilis* (Light and Clegg, 1974). The DNA concentration of *M. smegmatis* is lower under iron-deficient conditions, and it has been suggested that the increase in cell length is caused by either inhibition of DNA synthesis without proportional inhibition of cell growth, or by a direct effect on cell division or cell separation (Winder and O'Hara, 1962; Ratledge and Winder, 1964).

V Iron-Deficiency And The Regulation of Haem Synthesis

During iron-deficient growth of micro-organisms, haem synthesis is altered, as shown by the difference in cytochrome levels between iron-sufficient and iron-deficient cells (see previous section). Haem usually controls its

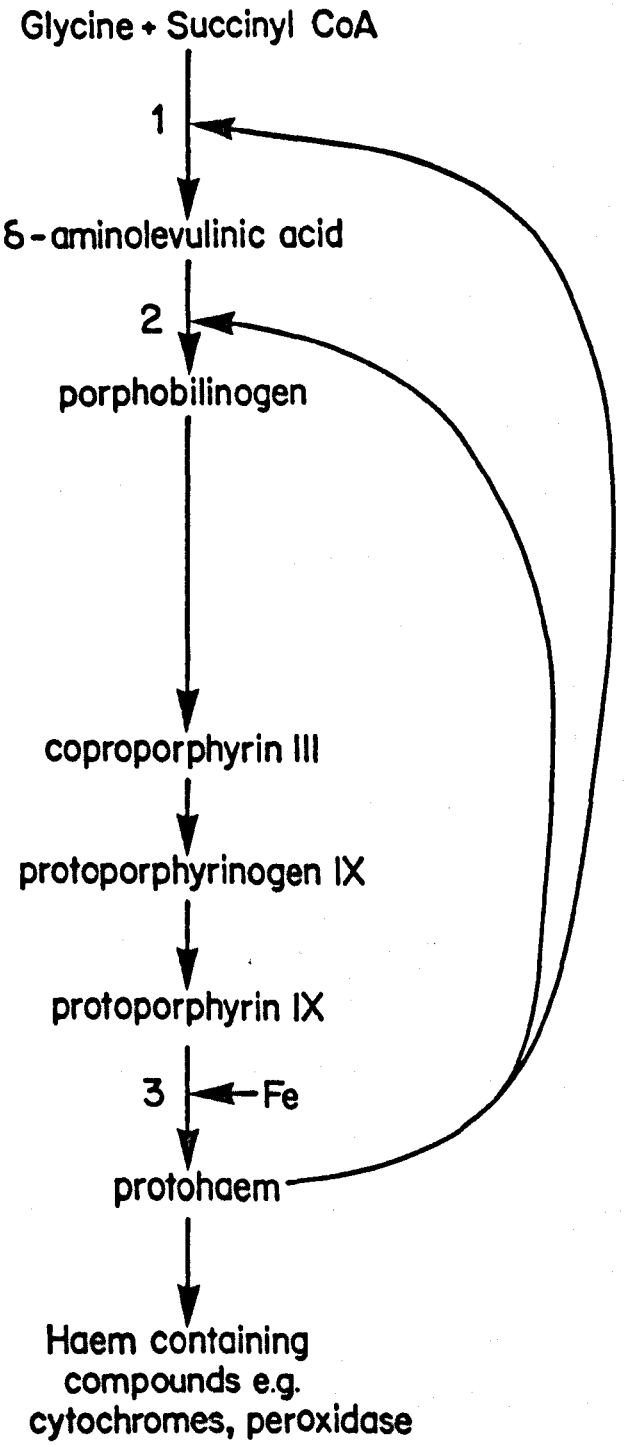
own synthesis by feedback inhibition of one or more enzymes of the synthetic pathway, but in the absence of iron to form haem, this control is relaxed. This relaxation usually results in the accumulation of porphyin by micro-organisms (Pappenheimer, 1947; Lascelles, 1962).

The control by haem is usually exercised at the level of δ-aminolevulinic acid synthetase, the first enzyme of the pathway (see Scheme 1), which catalyses the production of δ-aminolevulinic acid from glycine and succinyl-coenzyme A. Haem has been shown to both repress the synthesis and inhibit the activity of this enzyme in many organisms e.g. *Rhodopseudomonas spheroides* (Lascelles, 1975; Hatch and Lascelles, 1972; Warnick and Burnham, 1971); *Arthrobacter* (Kortstee, 1969); *Micrococcus denitrificans* (Tait, 1973[a,b]); *Propionibacterium shermanii* (Menon and Shemin, 1967), and *Rhizobium* (Cutting and Schulman, 1972). It was also thought that this was the site of control in *Spirillum itersonii* (Walker *et al*, 1967), but more recent work has shown this enzyme not to be affected by the iron-status of the cell in this organism (Ho and Lascelles, 1971).

In some organisms, control over the synthetic pathway is exercised at the level of δ-aminolevulinic acid dehydratase (see scheme 1). This, the second enzyme of the pathway, catalyses the production of porphobilinogen from δ-aminolevulinic acid. Control at this point is seen in *S. itersonii* (Ho and Lascelles, 1971; Lascelles, 1975); *Neurospora crassa* (Muthukrishnan *et al*, 1969, 1972) as well as *R. spheroides* (Yubisui and Yoneyama, 1972). However, control at this point is not as common as control of the first enzyme, δ-aminolevulinic acid synthetase.

Legend to Scheme 1: The enzymes δ-aminolevulinic acid synthetase, δ-aminolevulinic acid dehydratse, and ferrochelatase are represented by (1), (2) and (3) respectively in the scheme. Possible sites of feed-back inhibition are shown by arrowed lines.

Scheme 1. Outline Of Haem and Porphyrin Synthesis In Micro-Organisms.



In either case, the relaxed control due to the absence of iron to form haem, usually results in an overproduction of porphyin, which may be accumulated intra- or extracellularly. Some organisms may not accumulate porphyrin under these conditions e.g. *N. crassa*, which instead accumulates δ-aminolevulinic acid (Muthukrishnan *et al*, 1968). *M. smegmatis*, under iron-deficient growth conditions has been reported to have a stimulated production of porphrin (Winder and O'Hara, 1962) but the reverse was seen by Brown (1975), although admittedly with a different strain of *M. smegmatis*.

Little is known of the effect of iron-deficiency on the enzyme ferrochelatase, which is responsible for producing haem from porphyrin by the insertion of iron. No change in the activity of this enzyme has been seen in *N. crassa* under iron-deficient and iron-sufficient conditions, (Muthukrishnan *et al*, 1969), while this enzyme from *C. utilis* is reported to develop a lower K_m for iron under iron-deficient conditions (Jones and Jones, 1970). Although there is little evidence, it would not be surprising to see most porphyrin-accumulating micro-organisms follow the trend of *C. utilis*, whereby what little iron is available may be most effectively used for haem synthesis.

VI Hydroxamate - Compounds Produced During Iron-Deficient Growth of Micro-organisms

Micro-organisms produce a large number of iron-chelating agents in response to iron-deficiency, and these compounds may be conveniently divided into two classes. Firstly, the hydroxamates, which contain

substituted hydroxylamino groups, and secondly the phenolates (or catechols). Originally the term "siderochrome" was applied to the hydroxamates (Zahner *et al*, 1962), but it was suggested that this definition be enlarged to include the phenolate type compounds (Neilands, 1973). The term "siderophore" has now superseded the term siderochrome, in keeping with the function of these compounds in iron transport (Neilands, 1977) discussed in Sections VIII and IX.

The hydroxamates are a structurally diverse group of compounds, the common element of which is the presence of one or more hydroxylamino groups. Molecules containing three hydroxamic acid groups per molecule are the most common, and have been isolated from a wide range of sources (Neilands, 1974). Ferrichrome a cyclic hexapeptide, has been isolated from *Ustilago sphaerogena* (Neilands, 1952), *Aspergillus niger* and *Penicillium vesticulosum* (Keller-Schierlein *et al*, 1964). The basic structural features of the ferrichrome molecule form the basis for a whole family of iron-chelating agents, that includes coprogen, albomycin (an antibiotic), ferrirubin as well as several different ferrichromes (Neilands, 1973). Another large family of trihydroxamic acids, the ferrioxamines, have also been isolated from a wide variety of organisms including species of *Micromonospora, Streptomyces* and some *Actinomycetes* (Neilands, 1966, 1973). This family, like the ferrichrome group, differ in the side chains present on the parent molecule, that is found throughout the family. The basic structures of the ferrichrome and ferrioxamine groups are shown in figure 1.

Figure 1. Some Hydroxamic Acids Produced By Iron-Deficient Organisms.

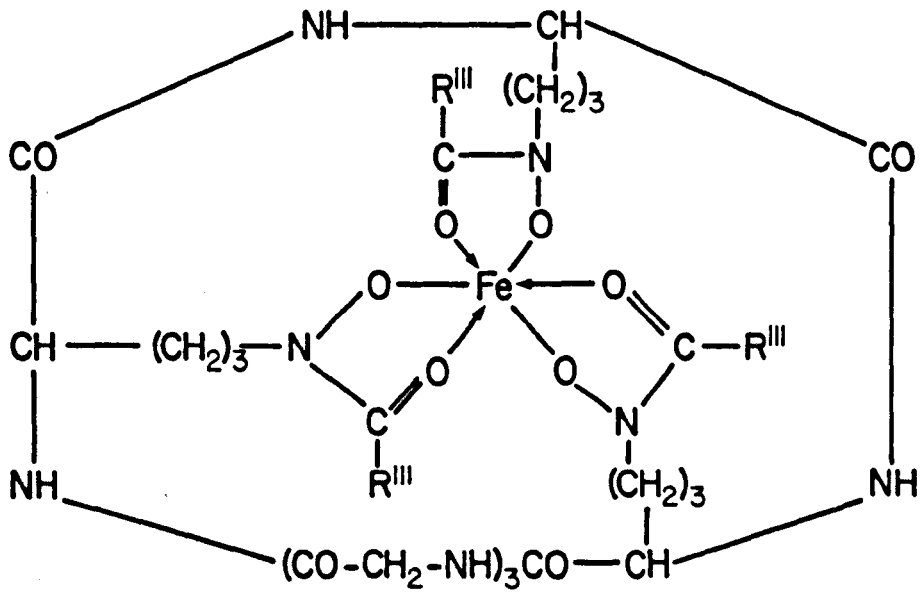
I Trihydroxamic Acids

a. Ferrichrome ($R^{III}=CH_3$)

U. sphaerogena,

A. niger,

P. vesticulosum.

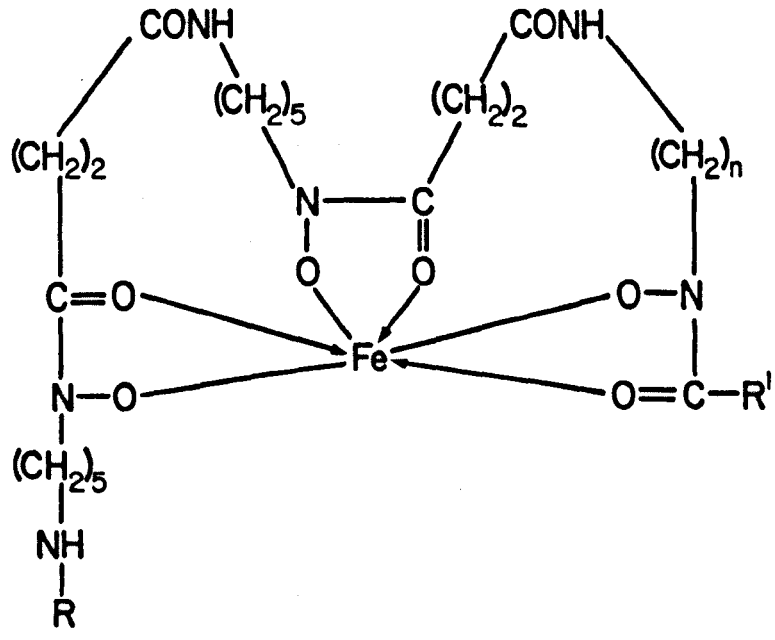


b. Ferrioxamine B (R=H,R'=CH,n=5)

Micromonospora spp.

Streptomyces spp.

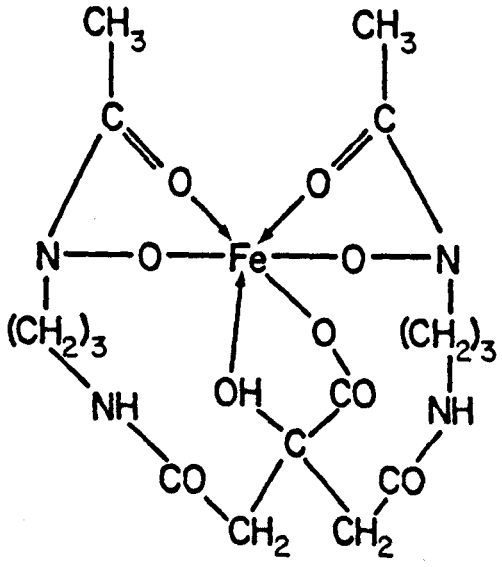
Actinomyces spp.



II Dihydroxamic Acids

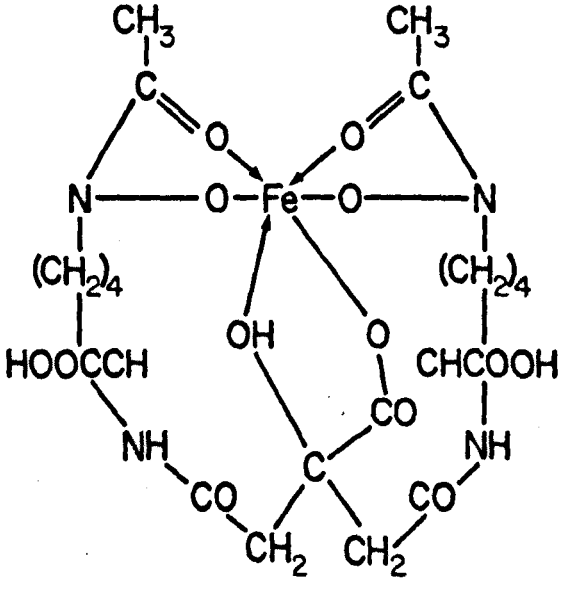
a. Schizokinen

B. megaterium.



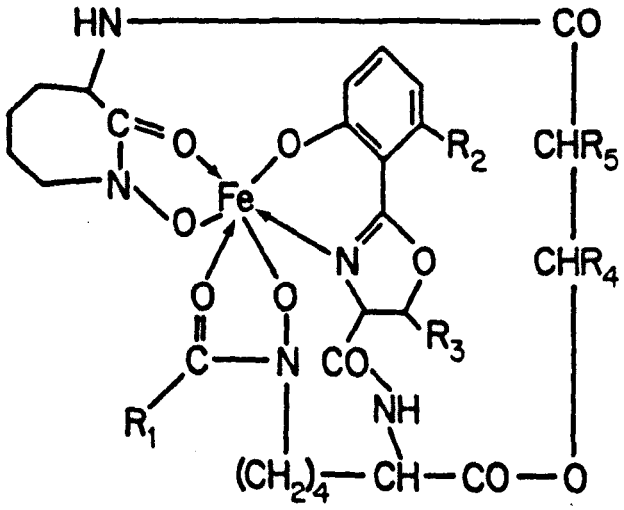
b. Aerobactin

A. aerogenes.



c. Mycobactin

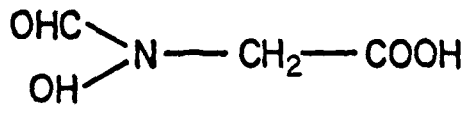
Mycobacteria spp.



III Monohydroxamic Acids

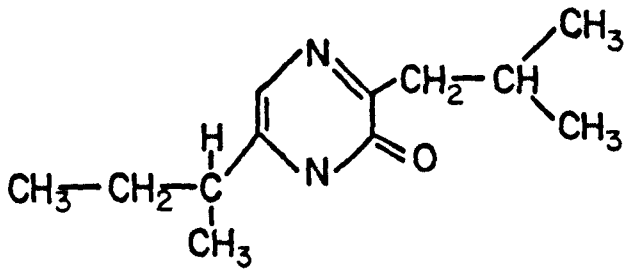
a. Hadicidin

Penicillium spp.



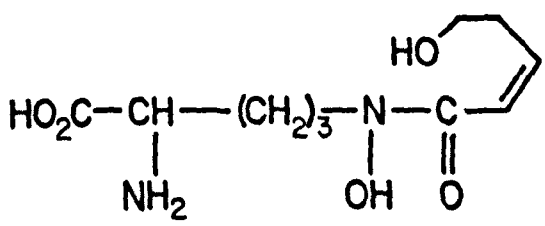
b. Aspergillic Acid

Aspergillus spp.



c. Fusarinine

Fusarium spp.



Dihydroxamic acids have also been isolated, e.g. schizokinen from *Bacillus megaterium* (Byers *et al*, 1967) and aerobactin, from *Aerobacter aerogenes* (Gibson and Magrath, 1969). Both schizokinen and aerobactin contain an internal residue of citric acid, which provides a single free carbonyl and hydroxyl group, both of which can co-ordinate to the bound iron. Another family of dihydroxamic acids, the mycobactins, have been isolated from most mycobacterial species (Snow, 1970). The only species of *Mycobacteria* so far examined that does not contain a mycobactin is *M. paratuberculosis*, which is discussed in Section XII. In addition to the two hydroxamate groups, mycobactin contains a phenolate and a substituted oxazoline group, which contribute an hydroxyl and a nitrogen respectively, to the co-ordination of the bound iron. Nocobactin, a compound similar to mycobactin, has been isolated from members of the genus *Nocardia* (Patel and Ratledge, 1973). The only difference between the nuclei of the two classes of compound is the presence of an oxazole ring in nocobactin, compared to an oxazoline ring in mycobactin (Ratledge and Snow, 1974). Mycobactin and nocobactin are unusual amongst the hydroxamates, as they are hydrophobic molecules, and located intracellularly. This may be in keeping with their proposed role in iron transport in these organisms (see Section IX).

Monohydroxamates have also been found, e.g. hadacidin, isolated from several *Penicillium* species (Dulaney and Gray, 1962), aspergillic acid, from species of *Aspergillus* (Dutcher, 1947) and fusarinine, from members of the genus *Fusarium* (Sayer and Emery, 1968). Fusarinine is the monomer

from which the trimer is built up whereby iron can be chelated, but it is not certain whether hadacidin and aspergillic acid chelate iron in this manner.

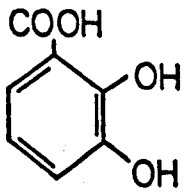
VII Phenolate Compounds Produced During Iron-Deficient Growth of Micro-organisms

A large number of phenolic compounds are produced during iron-deficient growth by micro-organisms. The first to be discovered was 2,3-dihydroxybenzoyl glycine, isolated from *Bacillus subtilis* (Ho and Neilands, 1958). 2,3-dihydroxybenzoate has been found in the iron-deficient growth medium of *B. subtilis* (Byers and Lankford, 1968), *Azotobacter vinelandii* (Corbin and Bullen, 1969) and *Streptomyces griseus* (Dyer *et al*, 1964). 3,4-Dihydroxybenzoate has also been isolated, from *B. anthracis* (Bakshi and Williams, 1969) and *B. cereus* (Cagle and Williams, 1969), while *M. phlei, M. smegmatis* and BCG produce salicylic acid or 6- methyl salicylic acid during iron-deficient growth (Ratledge and Winder, 1962, 1964).

Other conjugates of 2,3-dihydroxybenzoic acid have also been obtained. 2,3-dihydroxybenzoyl serine has been obtained from *B. subtilis* (Brot and Goodwin, 1968) and *E. coli* (Wang and Newton, 1969), while 2-N, 6-N- di (2,3-dihydroxybenzoyl)-L-lysine was obtained from *A. vinelandii* (Corbin and Bullen, 1969). The trimer of 2,3-dihydroxybenzoyl serine, termed enterochelin has been isolated from *Salmonella typhimurium* (Pollack and Neilands, 1970) along with *E. coli* and *A. aerogenes* (O'Brien and Gibson, 1970).

Figure 2. Some Phenolic Compounds Produced By Iron-Deficient Organisms.

a. 2,3 Dihydroxybenzoate

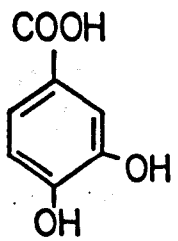


B. subtilis,

E. coli,

A. vinelandii.

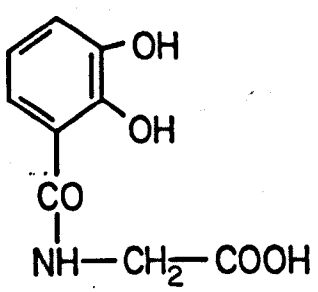
b. 3,4 Dihydroxybenzoate



B. anthracis,

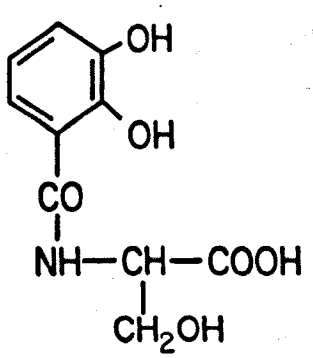
B. cereus,

c. 2,3 Dihydroxybenzoylglycine



B. subtilis.

d. Dihydroxybenzoylserine

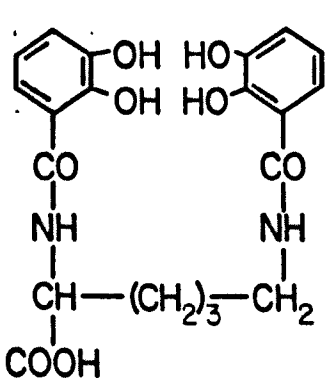


E. coli,

A. aerogenes,

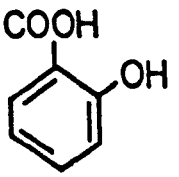
S. typhimurium.

e. 2-N,6-N-Di(2,3 dihydroxybenzoyl)-L-lysine



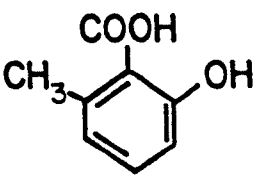
A. vinelandii

f. Salicylic Acid



M. smegmatis,
M. bovis var BCG,
Nocardia spp.

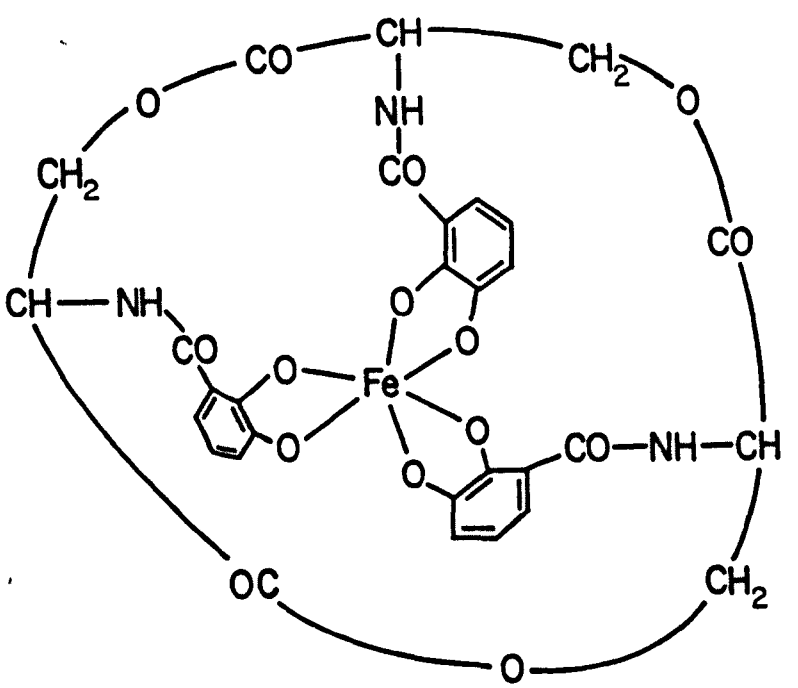
g. 6-Methyl Salicylic Acid



M. phlei.

h. Enterochelin

Enterobacteria.



However, apart from enterochelin (see Section VIII), the function of these compounds as iron-transporting or chelating agents *in vivo* is uncertain. Certainly salicylic acid is unable to hold iron in solution in the presence of phosphate ions (Ratledge *et al*, 1974) and its appearance in the culture medium may partly be the result of over-production during mycobactin synthesis, or from mycobactin degradation. However, work with an auxotrophic salicylate mutant of *M. smegmatis* which could not be satisfied by the addition of mycobactin instead of salicylate, has indicated that salicylate may have a function in *Mycobacteria*, other than as a precursor of mycobactin (Brown, 1975). Other members of this group, e.g. 2,3-dihydroxybenzoyl serine may simply be breakdown products of compounds involved in iron-transport although 2-N,6-N-di(2,3 dihydroxybenzoyl)-L-lysine has been suggested as having a role in transport (Neilands,1973).

VIII Iron Transport In Other Organisms

In *E. coli*, iron-transport is mediated by the ferri-enterochelin complex (Frost and Rosenberg, 1973), and after the entire iron-chelate complex is taken up into the cell, the iron is released by hydrolysis of the enterochelin, catalysed by the enzyme, ferri-enterochelin esterase (O'Brien *et al*, 1971). *E. coli* can also use citrate as an iron carrier (Cox *et al*, 1970; Langman *et al*, 1972) but this sytem is only found in cells grown on citrate (Cox *et al*, 1970). This organism can also take up iron by diffusion (Braun, 1978), as well as being able to utilise ferrichrome as an iron-transporting agent (Wayne and Neilands, 1975; Leong and Neilands, 1976).

Ferri-enterochelin uptake is an energy-dependent process, and transport is carried out via specific outer and inner membrane transport systems (Pugsley and Reeves, 1977; Hancock *et al*, 1977). The cell membrane was originally thought to be the only barrier to cell permeability, but the outer membrane is now thought to form a permiability barrier to compounds with a molecular weight greater than 500-600 (Decad and Nikaido, 1976). Several receptor proteins are believed to be involved in ferri-enterochelin uptake, whose functions involve coupling the energised state of the cytoplasmic membrane to the outer-membrane permeability and translocation of ferri-enterochelin across the cytoplasmic membrane (Hancock *et al*, 1977).

Other organisms e.g. *S. typhimurium* and *A. aerogenes* also have enterochelin-dependent iron transport systems, similar to that described for *E.coli* (Pollack *et al*, 1970; Wilkins and Lankford, 1970; Pollack and Neilands 1970; Rosenberg and Young, 1974). Also, both organisms have the ability to use other, hydroxamate-type, iron chelates for iron transport e.g. ferrichrome (Luckey *et al*, 1972; Leong and Neilands, 1976).

Iron transport in *B.megaterium* is mediated by the hydroxamate schizokinen (Davis and Byers, 1971; Arceneaux, and Lankford, 1971), but unlike enterochelin-mediated transport, schizokinen is not hydrolysed and is excreted back into the medium on the release of iron (Arceneaux, 1973). Ferri-schizokinen transport is energy and temperature dependent, and occurs via specific receptor molecules in the cell wall(Arcenceaux and Byers, 1976; Aswell *et al* 1977). The fungi *N.crassa* and *U.sphaerogena*, whose iron

transport is mediated by coprogen and ferrichrome respectively, possess iron-uptake systems similar to that of *B.megaterium* (Winkelmann 1974; Ernst and Winkelmann, 1977a; Emery, 1971). Another fungus, *F.roseum*, has a similar iron-uptake system to *B.megaterium*, via the hydroxamate fusarinine C (the trimer of fusarinine). However, this organism employs hydrolysis of the iron-chelate to release the bond iron (Moore and Emery, 1976; Emery, 1976).

IX Iron Transport In Mycobacteria

Iron was originally believed to be solubilised from the extracellular environment by salicylic acid, which is excreted into the medium in large amounts by *Mycobacteria* during iron-deficient growth (Ratledge and Winder, 1962; Antoine and Morrison, 1968). However, the discovery that salicylic acid could not hold iron in solution in the presence of phosphate (Ratledge *et al*, 1974) prompted the search for a new extracellular iron-chelating agent. A new compound was isolated, called exochelin, which was first extracted from the culture medium of iron-deficient *M. smegmatis* (Macham and Ratledge, 1975). A compound of similar chelating abilities has since been isolated from the culture medium of iron-deficient *M. bovis* var BCG (Macham *et al*, 1975). Exochelin is capable of holding iron in solution at physiological pH values, and is freely diffusible in both the ferri- and desferri- forms. It is also capable of solubilising iron from inorganic sources such as $Fe(OH)_3$ and $FePO_4$, as well as organic sources such as ferritin. Exochelin can also reverse the

inhibitory effect of serum on the growth of the *Mycobacteria*, and serum cannot prevent the transport of iron from ferri-exochelin, once formed, to the bacteria (Macham *et al*, 1975; 1977). Thus exochelin appears to fulfill the role originally attributed to salicylic acid.

Mycobactin has also been proposed as the extracellular iron-chelating agent (Kochan, 1973), but it is only detected in the growth medium of *Mycobacteria* when they are grown in the presence of a surfactant, such as Tween 80. In the absence of such a surfactant, no mycobactin can be detected in the mycobacterial growth medium (Ratledge and Marshall, 1972; Macham *et al*, 1975). This, and other properties such as the insolubility of mycobactin in water, and the inability to acquire iron from precipitated inorganic forms, have discounted mycobactin from performing the function of an extracellular iron-chelating agent.

The structure of exochelin has not yet been fully elaborated, but exochelin MS (the exochelin from *M.smegmatis*) is believed to contain 3 moles ε-N-hydroxylysine, 1 mole β-alanine and 1 mole threonine per mole of exochelin (L.P. Macham and M.C. Stephenson, unpublished results). Exochelin MB however, (the exochelin from *M.bovis* var BCG) contains a salicylic acid residue in place of β-alanine which gives this molecule an increased lipophilicity, compared to exochelin MS, as demonstrated by its extraction into chloroform as a method of purification (Macham *et al*,1975). Originally, it was thought that this lipophilicity would enable the exochelin to approach and interact with the cell surface, where the transfer of iron to mycobactin could occur (Macham *et al*, 1975). However, the finding

that mycobactin is not involved in iron transport from ferri-exochelin has discounted this theory (see below).

Transport of iron across the cell wall is mediated by mycobactin when iron is presented to *Mycobacteria* as ferri-salicylate (Ratledge and Marshall, 1972), and it was originally believed that the same mechanism applied when ferri-exochelin was presented to the cell (Macham *et al*, 1975). Iron would then be unloaded from the ferrimycobactin at the inner surface of the cell wall by the reduction of the iron-chelate, catalysed by the enzyme ferrimycobactin reductase (discussed in the next section). This would make *Mycobacteria* unusual in microbial systems, as there would be two iron-transporting agents, i.e. mycobactin and exochelin, involved in iron-uptake, as opposed to one in most other organisms. However, the thick, lipid-rich cell wall that is characteristic of the *Mycobacteria* would act as a barrier to hydrophilic molecules such as exochelin, thus necessitating the presence of a second lipophilic compound, to facilitate the entry of the iron into the cell. However, the recent discovery that mycobactin is not involved in iron-uptake when iron is presented to the cell as ferri-exochelin (Stephenson and Ratledge, 1979) invalidates this arguement, and questions the function of mycobactin *in vivo* (see Discussion).

X Intracellular Release Of Iron In *Mycobacteria*

The mechanism for the release of iron from ferri-mycobactin at the inner surface of the cell envelope was originally shown by Ratledge and Marshall (1972) to be mediated by an NADH-linked reductase in *M. smegmatis*.

Using NADH as a cofactor, this enzyme reduces ferri-mycobactin to ferro-mycobactin, and as mycobactin has little affinity for ferrous ions, the complex dissociates. This mechanism has the added advantage of leaving iron in a form suitable for insertion into haem precursors or non-haem iron proteins. Anaerobicity was found to be essential for the assay of this enzyme, to prevent the reoxidation of the ferrous ions, and their subsequent binding with mycobactin.

The enzyme has been further studied, and has been shown, contrary to expectations, to be a soluble and not a particulate enzyme (Brown and Ratledge, 1975). Presumably, this must mean that this enzyme is only loosely attached to the cell wall, if at all, which is surprising when considering that the substrate for this enzyme is located in the cell wall. However, the mechanism for insertion of iron into haem and non-haem compounds has yet to be established in *Mycobacteria*, but it is possible that it is not membrane bound. The finding that 65% of the total iron within *M. phlei* was recovered in the soluble fraction of the cell, rather than membrane bound (Kurup and Brodie, 1967), may support the finding that ferri-mycobactin reductase is not membrane bound.

The enzyme is strongly inhibited by sulphydryl reagents e.g. iodoacetate, which produce approximately 75% inhibition when present at 1mM. Sodium amytal, an inhibitor of NADH oxidation, is also a potent inhibitor, producing 80% inhibition when at 1mM (Brown and Ratledge,1975). The enzyme has a sharp pH profile, with optimum activity at pH7.0, but has only 40% of this activity at pH7.4, and

75% at pH6.5 (Brown and Ratledge, 1975). An unusual observation was that this enzyme has the same activity under both iron-deficient and iron-sufficient conditions, which is surprising considering that the concentration of mycobactin in iron-sufficient cells is undetectable. However, it has been suggested that the concentration of mycobactin in iron-sufficient cells, albeit very low, is enough to saturate the inner cell wall interface. In iron-deficient cells, although the concentration of mycobactin is much higher, the concentration at the inner cell wall interface will be the same (the majority of the mycobactin being located in the bulk of the cell wall), thus effectively presenting the cell with the same iron concentration under the two growth conditions (Brown, 1975).

A similar enzyme has been discovered in *U. sphaerogena*, by Komai and reported by Neilands (1973), although the specificity of this enzyme has been questioned. Similar enzymes have also been reported by Ernst and Winkelmann (1977b), from both *N. crassa* and *A. fumigatus*. Like the enzyme from *M. smegmatis*, this enzyme requires anaerobicity for assay, and is inhibited by sulphydryl reagents. However, the enzymes reported here are present at only 30% of the activity in iron-deficient cells, when assayed in iron-sufficient cells (Ernst and Winkelmann, 1977b).

Reduction of iron as a prominent feature of uptake has been implicated in *S. typhimurium* and *E. coli* (Leong and Neilands, 1976) although these studies involved the use of siderochromes other than those naturally produced by these organisms. This may reflect the ability of the enteric bacteria, such as *E. coli*, to utilise

the iron-transporting agents produced by other bacteria that occupy the same environment. *B. megaterium* also appears to use reduction as a means of releasing iron from its chelate, as the transporting agent (schizokinen) is excreted intact, back into the culture medium on the release of iron (Arceneaux *et al*, 1973). Other than these examples, no reports of a similar enzyme have appeared. However, it would not be surprising to find that reduction of iron was a common mechanism in microbial systems for removing iron from its chelate. This method is energetically favourable, as the chelating agent is free to bind more iron, while the iron itself is in a form ready to insert into suitable acceptor molecules.

The recent finding in our laboratory by Stephenson and Ratledge (1979) that mycobactin is not involved in iron uptake when iron is presented to the cell as ferri-exochelin, casts a serious doubt as to the part played by ferrimycobactin reductase in iron-uptake in *Mycobacteria*. This has led to the examination of the non-specific nature of this enzyme, which is discussed later in this thesis (see Discussion).

XI *Mycobacterium avium-intracellulare*

Mycobacterial taxonomy for many years, was a poorly defined area, consisting of *M. avium, M. bovis, M. tuberculosis* and a few saprophytic acid-fast bacilli (Wilson and Miles, 1946). The genus grew however, with many species being added to its numbers that were designated as "atypical". Runyon was the first to attempt to provide a systematic classification for this genus, to try and organise these

new *Mycobacteria* of clinical and microbiological importance (Runyon, 1959; Timpe and Runyon, 1954). Classification was based on pigment formation and speed of growth, giving the now well-known four Runyon groups:-

I Slow growing photochromogens

II Slow growing scotochromogens

III Slow growing nonphotochromogens

IV Rapidly growing *Mycobacteria*.

Within these groups, species were classified by standard microbiological tests e.g. colony morphology, temperature of growth, pH for growth. However it became apparent that these tests were not rigorous enough, as problems arose with overlaps between species e.g. *M. terrae* and *M. nonchromogenicum*, *M. gastri* and *M. kansii*. Numerical analysis now became an important tool, i.e. the examination of a group of organisms using a large number of characters in an attempt to correlate the results of a wide variety of microbiological and biochemcial tests, first suggested by Sneath (1957). This, and the use of more subtle techniques e.g. immunodiffusion, seroagglutination, has enabled many of these intergrades between species to be clarified.

One such group about which there is still confusion is that containing *M. avium* and *M. intracellulare* (also known as the Battey Bacillus). The majority of evidence suggests that *M. avium* and *M. intracellulare* are the same species, but Runyon (1967) has argued that *M. intracellulare* is a separate species on the basis of a number of characters including host range, the kind of disease produced, disease distribution and source of

infection. Wayne (1966, 1967) proposed that the two species are synonymous, using a numerical taxonomic approach, based on 25 characters from 17 tests. This finding was reiterated by Tsukamura and Mizuno (1968), who used a numerical analysis in conjunction with a "Hypothetical Mean Organism" prepared for each species under study. The study was performed using 97 characters on 754 strains, and a group of strains having greater than 90% correlation with the Hypothetical Mean Organism for a species, were defined as belonging to that species.

To co-ordinate the efforts of mycobacteriologists to resolve this and similar taxonomic problems, the InternationalWorking Group on Mycobacterial Taxonomy (I.W.G.M.T.) was established. Meissner *et al* (1974), as part of this group, proposed after a numerical analysis that *M. intracellulare* be reduced to a synonym of *M. avium*. This report, carried out in 18 laboratories used 118 characters on 59 strains, on which basis the strains were separated into species. The characters involved included the activity of various enzymes e.g. phosphatase, Tween hydrolase, growth at various temperatures, colony morphology, resistance to antibiotics, lipid analysis and pathogenicity. However even here a minority of the workers involved could not agree with the above proposal, and a minority report suggested that the term *M. avium-intracellulare* complex be retained until further evidence was acquired.

Using t.l.c. of mycobacterial lipids, a variety of workers have proposed that *M. avium* and *M. intracellulare* are the same species (Marks *et al*, 1971, Jenkins and Marks,1973;

Sehrt *et al*, 1976). This technique has been used in the classification of *Nocardia* and related bacteria (Minnikin *et al*, 1977a) and *Actinomadura* species (Minnikin *et al*, 1977b), which are closely related to the *Mycobacteria*. The above findings are supported by Stanford (1973) using immunodiffusion analysis, who proposed that, at best, *M. intracellulare* could only be given sub-specific status. Kubica *et al*, (1972), as part of the I.W.G.M.T. have also agreed that *M. avium* and *M. intracellulare* should be regarded as the same species, on the basis of a numerical analysis. Recently catalase has been used as a taxonomic tool (Wayne, 1978) by preparing the antigen to the enzyme from various strains, and cross-reacting the antigen with the enzyme from other strains. Although this technique has demonstrated that the two species are similar, the author has indicated that more work is necessary before the problem is resolved.

The additional problem exists of the apparent overlapping of this group with the species *M. scrofulaceum*. However, workers indicate that this species is synonymous with *M. avium* (Tsukamura *et al*, 1969; Marks *et al*, 1971; Wolinsky and Schaefer, 1973).

The possible use of mycobactin as a taxonomic tool has been discussed by Snow (1970). Mycobactin, the iron-chelating agent produced intracellularly during iron-deficient growth, differs from species to species on the basis of the side chains on the parent molecule (see figure 3). It has been proposed that these species-dependent differences can be exploited for taxonomic purposes (Snow, 1970). T.l.c. of mycobactins from various species has shown that these

differences are sufficient to give reasonable separation using this technique, except for mycobactins F and H (Snow and White, 1969). However, at this time, this method has not been used for taxonomic analysis, presumably due to the large mass of cells required to obtain enough mycobactin.

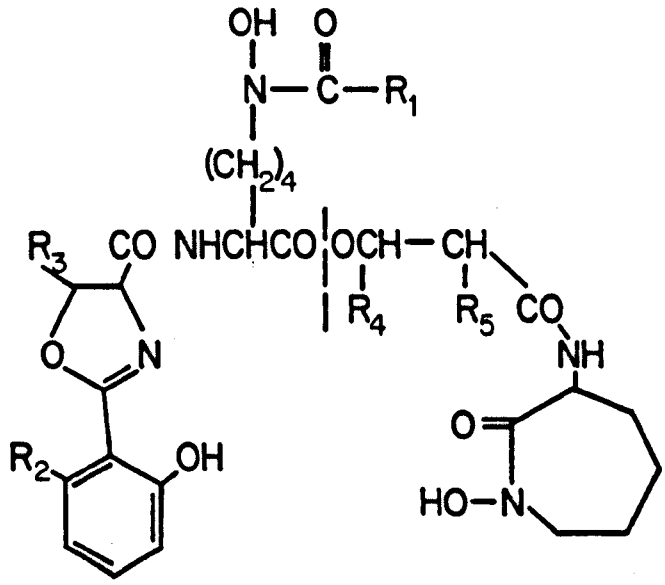
Thin-layer chromatography as a taxonomic tool has been applied to the genus *Nocardia*, which is similar to the *Mycobacteria*. Under iron-deficient conditions, *Nocardia* species produce an intracellular iron-binding compound called nocobactin, which is similar in structure and function to mycobactin (Patel and Ratledge, 1973). Nocobactins have been used in a taxonomic study of the *Nocardia*, the results of which correlated well with conventional taxonomic studies (Ratledge and Patel, 1976), indicating the reliability of this approach to a taxonomic study.

The technique of n.m.r. spectroscopy has also been employed on purified mycobactins from various sources and is able to identify the mycobactins on the basis of the characteristic spectrum produced (Greatbanks and Bedford 1969). Although proposed as a possible method of taxonomic analysis in the *Mycobacteria* (Snow, 1970), it has not been used at this time.

XII *Mycobacterium paratuberculosis*

M. paratuberculosis, formerly known as *M.johnei*, has for some time been recognised as the agent responsible for a chronic enteric condition in cattle and other ruminants,

Figure 3. Variation Of Mycobactin Structure With Species.



Species	Mycobactin Produced	R_1	R_2	R_3	R_4	R_5
M.phlei	P	19,<u>17</u>,15,cisΔ^2	CH_3	H	C_2H_5	CH_3
M. smegmatis	S	19,<u>17</u>,15,13 cisΔ^2	H	H	CH_3	H
M.tuberculosis	T	20,<u>19</u>,18,17, 20,<u>19</u>,18,17,Δ^2	H	H	CH_3	H
M.marinum	M	H	H	CH_3	18,17,16,15	CH_3
M. marinum	N	H	H	CH_3	18,17,16,15	CH_3
M.fortuitum	F	<u>17</u>,15,13,11, <u>9</u>,Δ^2	H	CH_3	CH_3	H
M. fortuitum	H	<u>19</u>,<u>17</u>,Δ^2	CH_3	CH_3	CH_3	H

Alkyl chains at R_1 and R_4 have the number of carbon atoms shown, with double bonds indicated. Figures underlined represent the most abundant homologue.

The portion of the molecule to the right of the broken line is the cobactin fragment of the molecule.

first known as Johne's Disease. This disease, at present, is spread throughout the world and is considered in England to be the most economically important infectious disease of cattle (Anon, 1975). The disease and its effects in ruminants are well documented (Larsen, 1972; Gilmour, 1976).

The source of infection is usually bacteria-laden manure, the organism remaining viable for long periods of time (at least six months) under atmospheric conditions that include freezing and drying (Larsen, 1972; Jørgensen, 1977). In the first months after infection, the organism multiplies in the intestinal mucosa and related lymph nodes, which leads to the development of minor lesions and the excretion of *M. paratuberculosis* in the faeces. The minor lesions then may extend and become confluent, causing interference with protein absorption and sometimes a 'protein leak' into the gut. There may be large numbers of *M. paratuberculosis* in the lesions, but usually at some stage the organism is disseminated throughout the body. In pregnant cows, the foetus may also become infected.

Unfortunately, little is known about the organism itself due to its fastidious growth requirements *in vitro*. Originally this organism could only be cultivated *in vitro* by the addition of heat-killed *Mycobacteria*, or extracts prepared from them, to the growth medium (Twort and Ingram, 1913). The active component for the culture of *M. paratuberculosis* was isolated from *M. phlei*, which was purified and called mycobactin P (Francis *et al*, 1953).

Mycobactins were subsequently isolated from other mycobacterial species, all of which have been examined as growth-promoting agents for *M. paratuberculosis* (Wheater and Snow, 1966; Snow and White, 1969); slight differences in the growth-promoting properties of the various mycobactins have been reported (Snow, 1970). Mycobactin S appears to be the most effective, showing significant stimulation of growth when added to the growth medium at 0.3µg/ml, while mycobactin R appears to be the least effective, being required at 3µg/ml. However a mycobactin concentration of 30µg/ml is needed with all mycobactins to produce maximal growth of *M. paratuberculosis* (Snow, 1970). The structures of the various mycobactins are shown in figure 3.

The dependence upon mycobactin for growth implies a deficiency in the production of mycobactin by the organism, which in turn may indicate an incompetence in iron-uptake. Morrison (1965) reported that the mycobactin requirement of *M. paratuberculosis* could be circumvented during growth at pH5.0-pH5.5. This could be due to the solubility of iron, which is 10^{-15} M at pH7.0, being 10^{-9} M at pH5.0. This million-fold increase in the solubility of iron may make it soluble enough not to limit the growth of *M. paratuberculosis* at this pH. If this were true, then simple diffusion would enable the organism to acquire enough iron for growth, thereby circumventing the mycobactin requirement. In support of this, it is known that *M. paratuberculosis*, as well as other *Mycobacteria*, grow *in vivo* in the phagosomes of the macrophages in their host. The pH in this environment is likely to be

between 5.0 and 5.5, thereby alleviating the organism's requirement for mycobactin (Wheeler and Hanks, 1965; Hanks, 1966). This would certainly account for the growth of *M. paratuberculosis in vivo,* where there would not, presumably, be a constant supply of mycobactin. However, Snow (1970) has postulated that there would be enough *Mycobacteria* being ingested with both soil and foodstuff, apart from those non-pathogenic *Mycobacteria* already present in the host, to provide *M. paratuberculosis* with a sufficient amount of mycobactin to support growth.

Phthiocol and other napthoquinones have been shown to stimulate growth of *M. paratuberculosis* (Woolley and McCarter, 1940), while vitamin K incorporated at 1.7µg/ml can stimulate the growth of this organism (Coletsos, 1971). However, Francis *et al,* (1949) failed to observe any significant effects on growth when vitamin K-type materials, extracted from *M. tuberculosis,* were incorporated into the growth medium of *M. paratuberculosis.* Certainly if vitamin K or related materials were responsible for supporting the growth of *M. paratuberculosis in vivo,* one might expect paratuberculosis (Johne's Disease) to have spread through a much wider range of animals. Presumably if vitamin K has any effect, it would be in conjunction with one or more other factors common only to those ruminants that contract this disease. During the growth of this organism *in vivo,* there may be a wide variety of bacterial species present in its environment, and it is possible that the iron-binding compounds produced by these organisms may enable *M. paratuberculosis* to overcome its mycobactin dependence. However, attempts

to stimulate the growth of *M. paratuberculosis* using naturally occurring iron-binding compounds e.g. ferrichrome, coprogen, haemin, have not met with any success (Morrison, 1965).

Certainly if the mycobactin-dependence of *M. paratuberculosis* can be overcome *in vivo*, then it is possible that mycobactin-synthesising bacteria, when growing *in vivo*, may not synthesise mycobactin. Some members of other mycobacterial species are also mycobactin-dependent when grown *in vitro* e.g. mycobactin-dependent *M. avium*, and it would be of interest to determine whether *in vivo* grown bacteria such as these, synthesise any mycobactin. The finding that mycobactin may not be involved in iron-transport (Stephenson and Ratledge, 1979) may affect our interpretation of mycobactin-dependence in these organisms. Thus, also of interest would be to determine whether these organisms synthesise an exochelin when grown both *in vivo* and *in vitro*.

Abbeviations Used

The following abbreviations have been used in the text.

CCCP	-	carbonylcyanide-m-chlorophenyl hydrazone
DCPIP	-	2,6- dichlorophenolindophenol
DNP	-	2,4- dinitrophenol
NEM	-	N- ethylmaleimide
Tween 80	-	polyoxyethylene sorbitan mono-oleate
PMS	-	phenazine methosulphate

MATERIALS AND METHODS

Maintenance of Organisms

M. smegmatis N.C.I.B. 8548 was maintained on nutrient agar slopes, while all strains of *M. avium-intracellulare* and *M. paratuberculosis* were maintained on Lowenstein-Jensen slopes. Mycobactin-dependent strains were grown on Lowenstein-Jensen slopes with mycobactin, incorporated to give a final concentration of 1µg/ml. All stock slopes were held at 2°C, *M. avium-intracellulare* and *M. paratuberculosis* strains were supplied by Dr. A. MacDiarmid, Institute for Animal Diseases, Compton, Berkshire, U.K.

Growth of Organisms

M. smegmatis and *M. avium-intracellulare* were grown on liquid medium (Ratledge and Hall, 1971), which contained (g/l in all glass double-distilled water): KH_2PO_4, 5.0; L-asparagine, 5.0; and glycerol, 25.2.

Mycobactin-dependant *M. avium* and *M. paratuberculosis* were grown on Dubos Broth liquid medium (Merkal and Curran, 1974) which contained (g/l in all glass double-distilled water): NaCl, 0.17; KH_2PO_4, 1.0; Na_2HPO_4, 2.5; casein hydrolysate, 0.5; L-asparagine, 2.0; glycerol, 5.0, Tween 80, 0.2; sodium pyruvate, 4.1.

The pH of the basic medium and the Dubos Broth was adjusted to 6.8 with 10 M NaOH before autoclaving. Metal ions were removed from the media by autoclaving with 0.5%(w/v) alumina ("for chromatography" grade), for 15 minutes at 103.5 k.Pa, shaking while hot and allowing to cool slowly (Donald *et al*, 1952). The media was filtered through

Whatman No.541 filter paper, and the first 100ml discarded. The medium was then dispensed in 100ml portions into 250ml conical flasks (metal-free) which were autoclaved at 103.5 k.Pa. for 15 minutes.

Immediately before inoculation, the 100ml portions of medium were supplemented with Zn^{2+} (0.46µg/ml), Mn^{2+}(0.1µg/ml), Mg^{2+}(0.04µg/ml) and Fe^{2+}, 0.05µg/ml or 2.0µg/ml, for iron-deficient or iron-sufficient growth respectively.

An alternative medium for the growth of mycobactin-dependent *M. avium* and *M. paratuberculosis* was Sauton's Liquid medium (Willis and Cummings, 1952), which contained (g/l in single-distilled water): KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.5; citric acid, 2.0; L-asparagine, 4.0; ferric ammonium citrate, 0.05; glycerol, 28.0; Tween 80, 0.2.

The pH of Sauton's medium was adjusted to 7.4 before autoclaving.

Inoculation was performed by transferring a loopful of pellicle from a statically grown culture, to a sterile, metal-free, McCartney bottle, containing 4.0ml, all glass double-distilled water, and metal-free glass beads. An even suspension was obtained by shaking and inoculation was carried out using a sterile, metal-free pipette.

M. smegmatis was incubated at 37°C, on an orbital shaker, at 200 rev/min; all other cultures were grown in the same manner, or statically at 37°C or 30°C (for the soil isolates).

The numbers M1-M34 represent the numbers used for convenience in this thesis, based on the numbering system used by the co-workers in the study of the *M. avium-intracellulare* complex.

Cleaning of Glassware

All glassware was cleaned by the method of Ratledge and Winder (1962), to remove all metal ions. The glassware was soaked overnight in 2% KOH in methylated spirit, then rinsed six times with tap water. The glassware was then soaked overnight in 8$\underline{\underline{M}}$ HNO_3, then rinsed six times with all glass double-distilled water.

Preparation Of "Growth Factors"

Various compounds, used as possible "growth factors" to replace mycobactin for growth of mycobactin dependent *M. avium* and *M. paratuberculosis*, were sterilised by filtration through a presterilised 0.1μ Sartorious membrane filter, into a presterilised, metal-free McCartney bottle.

Preparation Of Iron For Shift-Up Experiments

Iron was used in three forms in the shift-up experiments: soluble, insoluble and ferri-exochelin. 'Soluble iron' was prepared by dissolving $FeSO_4$ in uninoculated culture medium (96.1mg $FeSO_4.7H_2O$ per 100ml of growth medium) and adding 4.0ml $\underline{\underline{M}}$ H_2SO_4 to ensure that the iron remained in solution. This was sterilised in the usual manner, and the appropriate amount transferred to iron-deficient cultures to bring the iron concentration to that of iron-sufficiency. 'Insoluble iron' was prepared in the same manner, only omitting the acid, thus ensuring that the iron precipitated on sterilising. Portions of the suspension were transferred as for 'soluble iron'. Ferriexochelin was prepared as described later in this thesis, and sterilised by passage through a

pre-sterilised 0.1μ Sartorious membrane filter. Appropriate amounts of ferri-exochelin were transferred to iron-deficient cultures in the same manner as above.

In the further shift-up experiments, iron was added as 'soluble iron' in the same manner as above, using 2.46g $FeSO_4.7H_2O$ per 100ml culture medium (= 5mg Fe/ml). On harvesting the cultures in these experiments, the cells were washed with 600μM HCl containing 50mM EDTA (2x10ml), then with water, until the washings were neutral. This method solubilises any inorganic iron that has precipitated, but cannot remove iron from ferri-mycobactin.

Harvesting Of Cultures and Determination of Cell Dry Weight

Cultures were harvested either by filtration through Whatman No. 1 filter paper and washed twice with distilled water, or by centrifuging at 5,000*g* for 10min at 2°C.

Dry weights were determined by filtration through scintered glass vials, washed twice with distilled water and dried to constant weight at 50°C over KOH at reduced pressure.

Extraction and Estimation of Mycobactin

Freshly harvested cells were resuspended in ethanol for 24h at room temperature. A saturated solution of $FeCl_3$ (in ethanol) was added to ensure that mycobactin was extracted as the ferric complex. The cells were removed by filtration, washed with ethanol, and the combined ethanolic extracts mixed with an equal volume of chloroform. This was washed with water and the aqueous phase, containing the excess $FeCl_3$ and ethanol, was discarded. The organic phase was rewashed with water, dried over annhydrous $MgSO_4$

and evaporated to dryness under reduced pressure. The residue was taken up in methanol, and the mycobactin concentration estimated spectrophotometrically at 450nm, using $A^{1\%}_{1cm}$ = 42.8 for mycobactin S (White and Snow, 1969).

Purification of Mycobactin

Mycobactin was purified by a modified form of the method of Ratledge and Snow (1974). The partially purified mycobactin (see above), after being evaporated to dryness, was taken up in the minimum volume of cyclohexane/butan-1-ol (6:1,v/v) and the volume made up to 100ml with cyclohexane. Alumina (neutral grade) was added until all the ferric mycobactin was absorbed; this was then successively washed with cyclohexane (2x50ml), light petroleum, b.p.60-80°C (2x75ml), toluene, (1x75ml) and diethyl ether, (3x50ml). All washings were discarded. The ferric mycobactin was eluted with chloroform, which was evaporated to dryness under reduced pressure and taken up in the minimum volume of ethanol. This was chromatographed on a column of Sephadex LH20 (3x35cm), which was washed with ethanol.

The mid-fraction of the eluted red material was evaporated to dryness under reduced pressure, and stored over KOH and paraffin-wax chips at reduced pressure. The purity of the mycobactin obtained by this method was greater than 90%, using $A^{1\%}_{1cm}$ = 42.8 (White and Snow, 1969).

Preparation of Desferrimycobactin

Ferrimycobactin, in $CHCl_3$, was washed with an equal volume of 6M HCl, and the $CHCl_3$ layer then repeatedly washed

with water until the washings were neutral. At this point, the $CHCl_3$ layer was washed with a further 50ml water, which was saved. The $CHCl_3$ layer was now washed with an equal volume of 6M HCl, and then washed using the 50ml water from above, until the washings were neutral.

Preparation of Aluminium Mycobactin

Aluminium mycobactin was prepared by the method of Snow (1965). Desferrimycobactin (1.26mg) was dissolved in butanol (0.2ml) to which was added annhydrous $AlCl_3$ (0.02ml of a 2% solution in ethanol, w/v). This was diluted with ethanol (0.5ml) and then washed with water until the washings were not acidic. The butanol was removed by evaporation under reduced pressure.

Nuclear Magnetic Resonance (n.m.r.) Spectroscopy of Mycobactins

N.m.r. spectroscopy of mycobactins was performed using the metal-free forms in $CDCl_3$ (2-10% w/v, according to the material available), with a JNM-PMX 60 n.m.r. spectrometer. The spectra were studied and the peaks identified by reference to the work of Greatbanks and Bedford (1969).

High Pressure Liquid Chromatography (h.p.l.c.) of Mycobactins

H.p.l.c. of mycobactins was performed using pure ferrimycobactins, dissolved in methanol (2-5% w/v, depending on the material available), with a Partisol O.D.S. column linked to a Varian 850 pumping unit and a Perkin-Elmer LC55 spectrophotometer with chart recorder. The solvent system employed was methanol/water (100:30, v/v) and a flow rate of 100ml/h was employed throughout.

Gas Liquid Chromatography (g.l.c.) Of The Fatty Acids From Mycobactin

Ferrimycobactin (2-10mg) was dissolved in methanol (1ml) and refluxed with 6M HCl (1ml) for 4h. The solution was extracted with diethyl ether (2x3ml) and the combined ether layers washed with water (1x3ml). The ether layer was evaporated to approximately 1ml, and the methyl esters of the fatty acids prepared by the addition of diazomethane until a yellow colour persisted. The methyl esters of the fatty acids were identified by their retention times on a column of diethylene glycol succinate (5mmx1.5m), on 80/100 mesh Supelcoport (Chromatography Services Co., Wirral, Merseyside). The carrier gas used was N_2, employed at a flow rate of 30ml/min throughout, at a column temperature of 175°C.

Mass Spectrometry Of Mycobactins

Mass spectrometry was performed on the aluminium mycobactins, using an MS9 double focusing mass spectrometer, with the solid-sample insertion probe at 70eV and a temperature of 200°C.

Degradation of Mycobactin And Isolation Of Cobactin

Mycobactin was degraded by the method of Snow (1954) in order to isolate the cobactin fragment of the molecule (Fig. 3). Desferrimycobactin (0.5g) was dissolved in methanol (10ml), 10ml cold M NaOH was added, and the solution left on ice for 45 min. 10ml M H_2SO_4 was then added with stirring (a precipitate may form), followed by 10ml water, and the suspension was extracted with ether (3x25ml), which dissolved any precipitate that had formed.

The ether extracts were washed with water (2x5ml), and the aqueous material pooled, and evaporated to dryness. The residue from the aqueous solution was extracted with ethanol (3x50ml) by grinding in a mortar and filtering, and the filtrate evaporated. The residue was again taken up in ethanol (30ml) and the solution filtered; the filtrate was evaporated leaving an almost white residue (cobactin).

The cobactin was purified by dissolving the sample in methyl ethyl ketone (10ml) and refluxing for 10 min in the presence of activated charcoal (Norit GSX). After cooling, the solution was filtered, the filtered material washed with more solvent, and the combined filtrates evaporated to dryness under reduced pressure.

Determination of the Redox Potential of Ferrimycobactin

The redox potential of ferrimycobactin was determined using the method of Rooney (M. Rooney, personal communication to C. Ratledge). The reduction voltage of the ferri-ferromycobactin couple was determined by differential cathode ray polarography. A ferrimycobactin solution (1.5ml of a 0.1%(w/v) solution in ethanol, plus 3.5ml Tris/HCl, 100mM, pH8.0) was subjected to a series of voltage sweeps using a Davis polarograph. A similar solution, minus any mycobactin, was incorporated into the "blank" for this instrument. During the voltage sweep, the point at which maximum current was observed to flow represented the voltage at which maximum reduction of ferri to ferromycobactin occurred. This was observed as a 'peak' in the voltage sweep, traced on the cathode

ray oscilliscope, and was recorded using a polaroid camera attachment. From this, the reduction voltage was determined.

The redox potential of ferrimycobactin was found from a calibration graph of reduction voltage against redox potential for a series of iron-containing compounds. The reduction voltage of these compounds of known redox potential was determined in the same manner as that for ferrimycobactin.

Preparation of Ferriexochelin MS

The culture medium from freshly filtered 60h *M. smegmatis*, grown under iron-deficient conditions was stirred for 1h after the addition of enough $FeCl_3$ (a saturated aqueous solution) to give the spent growth medium a pale yellow colour. The culture fluid was then filtered through a Whatman No. 1 filter, and the filtrate pumped on to a Ze olite SRC225 column in the Na^+ form (3x10cm). The column was washed with water and the ferriexochelin eluted with $1\underline{M}$ NH_3/NH_4Cl pH9.5. The collected coloured fractions were pooled, the volume reduced by evaporation under reduced pressure, and desalted by passage through a Sephadex G10 column (4x50cm).

Isolation of Exochelins From *M. avium-intracellulare*

The spent culture medium from freshly filtered *M. avium-intracellulare* grown iron-deficiently was treated with $FeCl_3$ in the same manner as used in the preparation of exochelin MS (see above). However, these exochelins were different from exochelin MS, as they could be extracted into chloroform, in the same manner as exochelin MB (see Introduction). After filtration through a Whatman No. 1 filter, the culture fluid was extracted with

chloroform (3x100ml), and the chloroform layer washed with water (1x50ml). The chloroform layer was dried over $MgSO_4$, then evaporated to dryness under reduced pressure.

Attempted Isolation Of Exochelin and Salicylic Acid From *M. paratuberculosis*

The spent growth medium from iron-deficiently grown *M. paratuberculosis* (and other mycobactin-dependent *Mycobacteria)* grown on the growth factor Nocobactin 3318 (see Results) was adjusted to pH7.0, and stirred for 1h after the addition of enough $FeCl_3$ (a saturated aqueous solution) to give it a pale yellow colour. The culture fluid was filtered through Whatman No. 1 and extracted with $CHCl_3$ (3x200ml). The $CHCl_3$ layer was washed with water (1x50ml) and dried over $MgSO_4$. The $CHCl_3$ layer would contain any exochelin that had been produced (if it was chloroform soluble) as well as possibly containing nocobactin 3318 (the nocobactin from *Nocardia asteroides* N.C.I.B.3318 used to promote growth).

The combined aqueous material was adjusted to pH 2.5 using $\underline{M}$ H_2SO_4, and extracted with ether (3x100ml). The combined ether extracts were dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The residue, taken up in methanol, would contain any salicylic acid produced by this organism.

Identification Of Salicylic Acid

Salicylic acid was identified by its u.v. spectrum in methanol, and its mobility (R_F=0.70) on ascending paper chromatography in propanol-2-ol/water/NH_3 (8:1:1 by vol).

Preparation of Cell-Free Extracts For Determination Ferrimycobactin Ferri-Reductase Activity

Freshly harvested *M. smegmatis* was mixed with an equal volume of 100mM Tris/HCl, pH7.0, at 2°C, and disrupted in a pre-cooled French Pressure Cell at 3.5×10^4 kN/m^2. The exudate was incubated with deoxyribonuclease II (20mg) at 2°C for 30 min with stirring, to reduce viscosity. The exudate was then centrifuged at 30,000*g* for 30min at 2°C, and the supernatant fluid used for assay.

Preparation of Cell-Free Extracts For Determination Of δ-Aminolevulinic Acid Synthetase Activity

1) Toluene Lysis: Freshly harvested *M. smegmatis* was resuspended in a toluene/ethanol mixture (1:4,v/v) in the ratio, 100mg wet cells to 100µl mixture. This was vigourously mixed for 5 minutes at room temperature, and the material used directly.

2) Freeze Drying and Grinding Freeze dried cells were made into a thick paste with alumina (polishing grade) and 50mM KH_2PO_4 pH7.0, and ground with a pre-cooled agate pestle and mortar for 5 min at 2°C. When the viscosity of the mixture changed, the mixture was centrifuged at 14,500*g* for 20 min at 2°C and the supernatant fluid used for assay. This method was repeated on fresh cells, incorporating 1% mercaptoethanol in the grinding buffer.

3) Ultrasonication: Freshly harvested cells, resuspended in 50mM KH_2PO_4 pH7.0, were ultrasonicated for 5x1 minute intervals, with 30 seconds cooling between each interval, at maximum output using a Dawe soniprobe. The suspension was centrifuged at 14,500*g* for 20min at 2°C, and the supernatant fluid used for assay. This method was repeated on fresh

cells, incorporating 1% mercaptoethanol in the buffer.

4) French Pressure Cell: Freshly harvested cells, resuspended in 50mM KH_2PO_4, pH7.0, were disrupted in a pre-cooled French Pressure cell, at 3.5×10^4 kN/m^2. The exudate was centrifuged at 14,500*g* for 20 min at 2°C, and the supernatant fluid used for assay. The method was repeated on fresh cells, using 1% mercaptoethanol and 5mM pyridoxal phosphate in the buffer. Also attempted was the harvesting and disrupting of the bacteria at room temperature, both in the presence and absence of pyridoxal phosphate and mercaptoethanol.

Preparation of Electron Transport Particles From *M. smegmatis*

Electron Transport Particles (E.T.P.) were prepared by the method of Brodie (1959). Freshly harvested cells were resuspended in an equal volume of 1.2M sucrose at 2°C, and sonicated using a Dawe Soniprobe at maximum output, for five one-minute intervals, with thirty seconds cooling between each interval. The suspension was centrifuged at 20,000*g* for 30 minutes at 2°C, and the supernant fluid adjusted to pH7.4. This was then centrifuged at 50,000*g* for 30 minutes at 2°C.

The supernatant was centrefuged at 140,000*g* for 90 minutes at 2°C in an MSE 65 superspeed centrifuge. The pellet obtained was resuspended in 100mM Tris/HCl, pH7.0, and centrifuged at 140,000*g* for 60 minutes at 2°C. The pellet was resuspended in 100mM Tris/HCl pH7.0, and used for assay.

Attempted Preparation Of Cell Ghosts from *M. smegmatis*

Cell Ghosts were prepared by the method of Asano *et al*, (1973). Cells grown for 36h were incubated with glycine

(1.55%) and sucrose (0.5M) for 3 hours, after which lysozyme and EDTA were added to give final concentrations of 2.5mg/ml and 5mM respectively.

The appearance of osmotically sensitive cells was followed turbidimetrically using an SP600 spectrophotometer. A sample of the cell suspension was diluted with 30 volumes of distilled water, while for control readings, an equal volume of cells was diluted with 30 volumes of 0.5M sucrose. The decrease in turbidity of the water-lysed samples compared to the control was used as a measure of cell lysis. When the difference between the control and the lysed samples remained constant, the incubation was terminated.

The sample was centrifuged at 5,900*g* for 10 minutes at 2°C, and the pellets homogenised in 2mM $MgCl_2$ at 2°C, (60ml per 10g cells, wet weight) in the presence of deoxyribonuclease II (2mg per 100ml of suspension). The ghosts were recovered by centrifuging at 5,900*g* for 10 minutes at 2°C, washed three times with distilled water and resuspended in 10mM $MgCl_2$.

Sucrose Density Gradient Centrifugation Of Ghosts

Centrifugation of ghosts was carried out through a discontinuous sucrose density gradient at 24,000*g* at 2°C for 17 hours. The gradient consisted of 4.0ml each of 1.30, 1.55, 1.65 and 1.75M sucrose, and 2.0ml of 2.0M sucrose. The ghosts appeared in the 1.55M and 1.65M sucrose, whereas intact cells sedimented at the bottom of the tube.

Determination of FerriMycobactin Ferri-Reductase Activity

Activity of ferrimycobactin ferrireductase (FMF Reductase) was determined by the method of Brown and Ratledge (1975). The assays were performed using silica anaerobic cuvettes, which contained, in a final volume of 3.0ml: - EDTA, 10μmoles; ferrimycobactin S in Triton 101, 400nmoles; NADH, 10μmoles (in side-arm, to initiate the reaction); Tris/HCl, pH7.0, 200μmoles; enzyme extract and distilled water to 3.0ml.

The cuvettes were gassed with O_2 free N_2 for at least 15 minutes, by using long hypodermic syringe needles fitted through self-sealing rubber caps. The cuvettes were incubated at 37°C for 10 minutes before initiating the reaction with NADH. The reaction was monitored by following the decrease in absorbance at 450nm, using an SP1800 recording spectrophotometer, using a blank that lacked NADH.

Determination of Succinate Dehydrogenase Activity

Succinate dehydrogenase activity was determined by the method of Brodie (1971). The assay contained, in a final volume of 3.0ml; Tris/HCl, pH7.4, 50μmoles; KCN, 20μmoles; P M S , 3μmoles; DCPIP, 15μmoles; sodium succinate, 50μmoles; cell extract and distilled water to 3.0ml.

The assay was performed in silica cuvettes and was followed at 600nm using an SP1800 recording spectrophotometer, at 25°C. The reaction was initiated by the addition of succinate.

Determination of δ-Aminolevulinic Acid Synthetase Activity

1) δ-Aminolevulinic Acid (δALA) synthetase activity was assayed by the method of Burnham (1970). The assay contained,

in a final volume of 1.0ml:- Tris HCl,pH7.8, 400μmoles; succinyl coenzyme A, 600nmoles; glycine, 200μmoles; pyridoxal phosphate, 200nmoles; enzyme extract and distilled water to 1.0ml.

The reaction mixture was incubated at 37°C for 10 minutes prior to initiation with succinyl coenzyme A. The reaction was terminated by the addition of 1.0ml of 10% trichloroacetic acid. The assay mixture was centrifuged for 5 minutes at 2,000*g* in an M.S.E. bench centrifuge. The supernatant fluid was assayed for the presence of δ-ALA by boiling for 15 minutes with 2.0ml 4 M sodium acetate buffer pH4.2, and 0.05ml acetyl acetone. After 5 minutes cooling, 3.5ml of Ehrlich's reagent, (consisting of 1.0g dimethylamino-benzaldehyde, 18ml perchloric acid (70%), and made up to 50ml with glacial acetic acid), was added to the assay mixture. The absorbance at 555nm was read after 15 minutes.

2) δALA Synthetase activity was also determined by the radiochemical method of Irving & Elliott (1970). The assay was carried out in centrefuge tubes, in a shaking water bath at 37°C. The assay consisting of: Tris/HCl, 2.5M pH7.4, 10μl; glycine, 1.25M 40μl; sodium succinate, M 5μl; [1,4,^{14}C]-succinic acid, 5.76 mM, sp.act. 8.68μCi/μmole, 50μl; $MgCl_2$, 1M, 10μl; coenzyme A, 8.5mM, 25μl; glutathione, 40mM, 25μl; pyridoxal phosphate, 20mM, 25μl; ATP, 0.5M, 25μl; sodium arsenite, 0.25M, 10μl; sodium-D/L-malate, 0.25M, 10μl; sodium malonate, 0.5M, 10μl; Antimycin A, 50μg/ml, 25μl; succinate thiokinase, sp. act. 9.6μmoles/ml, 50μl; enzyme extract and distilled water to 0.5ml. The

pH of all solutions was brought to 7.4 using Tris-base.

The assay was terminated by the addition of 2.0ml, 0.3M trichloroacetic acid. Also added were 1.5ml H_2O, containing 100µmoles unlabelled succinate and 1 mole δALA. This was centrifuged at 2,000*g* for 10 minutes in an M.S.E. bench centrifuge, and the supernatant fluid loaded onto a Dowex 50 column (3x1cm) in the H^+ form (8% cross-linkage, 100-200 mesh). The column was washed with 30ml distilled water (which removed any [^{14}C] - succinate), and the δALA was eluted using 10ml pyridine-acetate buffer, pH6.0. The column eluate was collected in a round-bottom flask containing 1 drop of concentrated hydrochloric acid, and evaporated to dryness (x2) under reduced pressure.

The residue was taken up in 2.0ml distilled water, and 1.8ml added to 1.0ml, 1M sodium-acetate buffer, pH4.7, plus 0.1ml acetyl acetone. This was heated in a boiling water bath for 15 minutes. After cooling, the solution was extracted with ethyl acetate (25ml), and the organic layer washed with 2-3ml distilled water, then evaporated to dryness. The residue was taken up in 1.0ml methanol and 100µl portions dispensed for liquid scintillation counting.

Determination of δ-Aminolevulinic Acid Dehydratase Activity

δALA dehydratase was assayed by the method of Mauzerall and Granick (1956). The assay contained, in a final volume of 3.0ml:- δALA (neutralised by Tris-base), 10µmoles; $MgSO_4$, 10µmoles; β-mercaptoethanol, 20µmoles; Tris/HCl,pH9.0, 100µmoles; enzyme extract and distilled water to 3.0ml.

The reaction mixture was preincubated at 37°C for 10 minutes, prior to the initiation of the reaction by the addition of δALA. The reaction was terminated by the addition of 1.0ml 20% trichloroacetic acid. The assay mixture was centrifuged at 2,000*g* for 5 minutes in an M.S.E. bench centrifuge, and the supernatant fluid assayed for porphobilinogen by the addition of 1.0ml Ehrlich's reagent. The absorbance at 555nm was read after 15 minutes.

Preparation and Assay Of Succinyl Coenzyme A

Succinyl Coenzyme A was prepared by the method of Simon and Shemin (1953). Coenzyme A (10mg) was dissolved in 0.5ml sodium bicarbonate buffer, 50m$\underline{M}$ pH8.0, and succinc annhydride was added (3mg, a 3 fold excess). The mixture was kept on ice with a constant stream of nitrogen blown across the surface. The pH of the mixture was then brought to 1.0 using 6$\underline{M}$ HCl, and the volume brought to 2.0ml with distilled water.

The succinyl coenzyme A was assayed by the method of Lipman and Tuttle (1945). To an aliquot of succinyl coenzyme A solution, diluted to 1.0ml, was added 0.5ml hydroxylamine solution (see below). After 15 minutes incubation at 30°C, 1.5ml of ferric chloride reagent was added, and the absorbance at 540nm measured immediately.

The hydroxylamine solution was prepared by mixing equal volumes of 28% hydroxylamine hydrochloride (w/v) and 14% sodium hydroxide (w/v). Ferric chloride reagent was prepared by mixing equal volumes of 5% $FeCl_3$ in 0.1$\underline{M}$ HCl (w/v) and a solution containing 1vol concentrated HCl plus 3vol 8% trichloroacetic acid (w/v).

The concentration of succinyl coenzyme A was found from a calibration curve prepared using succinic annhydride.

Extraction and Identification of Extracellular Porphyrin

Extracellular porphyrin was determined by the method of Tanaka (1968). The filtered spent growth medium was extracted with ethyl acetate/glacial acetic acid (3: 1v/v; 3x200ml). The organic phase was washed with water (1x200ml), and the porphyin extracted into 10%HCl. The concentration of the porphyin (µg/l) was calculated from the equation -

$$\left[2 \times A_{401} - (A_{430} + A_{380})\right] \times 837 \times \frac{v}{V}$$

where v = vol. HCl extract

and V = vol. of spent growth medium.

The porphyin obtained was identified by determination of the absorption spectrum in chloroform, using an SP1800 recording spectrophotometer. Identification was also by means of double development paper chromatography using:- ascending chromatography in kerosene/chloroform (2:1, v/v; Development 1), and ascending chromatography in kerosene/n-propanol (5:1, v/v; Development 2).

Chromatography was performed on Whatman No. 1 chromatography paper, using standard solutions of known porphyrins for comparison.

Extraction of Intracellular Porphyrin

Porphyrin was extracted from whole cells by the method of Falk (1964). Cells were incubated overnight in ethyl acetate/glacial acetic acid (3:1, v/v), filtered and washed

with more solvent. Combined solvent extracts were extracted with 10%HCl (2 vol), and the aqueous phase was brought to pH 4.0 using saturated sodium acetate. The aqueous phase was extracted with chloroform (2x1 vol), which was evaporated to dryness. The residue was taken up in a known volume of chloroform, and the absorption spectrum determined using an SP1800 recording spectrophotometer.

Intracellular Iron Determination

Total intracellular iron was determined by the method of Kurup & Brodie (1967). A portion of cells (200 - 300mg) was digested with 2.0ml nitric acid/perchloric acid (10:1, v/v) in a micro-Kjeldahl flask, until a colourless fluid remained.

After cooling, the residue was taken up using all glass double-distilled water, the pH brought to 4.0 using 10M NaOH, and the volume made up to 5.0 ml in an iron-free graduated flask using double-distilled water. To 3.5ml of this solution was added 0.5ml M sodium acetate buffer, pH4.0; 0.5ml 0.5% (w/v) o-phenanthroline in 50% ethanol(v/v); and 0.5ml 10% (w/v) hydroxylamine hydrochloride. The assay mixture was preincubated at 37°C for 5 minutes prior to initiation with o-phenanthroline. After 30 minutes at 37°C, the absorbance at 510nm was measured. All solutions were prepared using iron-free glassware and double-distilled water.

For every determination, a blank containing a suitable amount of resuspending buffer was digested in the same manner as was an internal standard containing 200µmoles Fe^{2+}.

Intracellular Non-Haem Iron Determination

Non-haem iron was determined by the method of Kurup and Brodie (1967). A disrupted cellular preparation was treated with sodium dithionite (0.5ml of a 4mg/ml solution, freshly prepared), before the addition of trichloroacetic acid (1.0ml of a 20% solution, w/v). The sample volume was made up to 4.0ml, before centrifuging the sample at 2,000*g* for 10 minutes in an M.S.E. bench centrifuge. The supernatant was assayed for iron in the same manner as above, omitting hydroxylamine hydrochloride from the assay.

Determination of Cytochromes As Their Pyridine Haemochromogens

Cytochrome concentrations were determined by the method of Jacobs and Wolin (1963). Freshly harvested cells were extracted overnight in acetone plus 1% 2.4 M HCl (10ml solvent to 300mg cells), filtered and washed with more solvent. The combined solvent extracts were extracted with 25ml, then 10ml of acidic ether (1vol 1.5 M HCl plus 4 vol diethyl ether), and the combined ether extracts washed with 30ml, then 20ml of 0.5 M HCl, and then 20ml 3% NaCl.

Ether was removed by passage of N_2, and the residue was taken up in 3.5ml pyridine and 3.5ml 0.2 M KOH.

The cell residue from filtration was resuspended in a solution of pyridine/0.2M KOH (1:1, v/v) by grinding in a hand operated tissue homogeniser. The absorption spectra were determined using a Cary 14 u.v. recording spectrophotometer.

Protein Estimation

Protein concentration was determined by the Biuret method (Gornall *et al*, 1949), using bovine serum albumin

fraction V as a standard. In the case of E.T.P. and cell ghosts from *M. smegmatis*, the membranes (0.25ml) were boiled with 0.75ml 1 M NaOH for 10 minutes prior to measuring the protein concentration. A separate calibration graph for this method was produced, using bovine serum albumin fraction V as a standard.

Measurement of Radioactivity

All radioactivity was measured by liquid scintillation counting. Aqueous samples were counted in scintillation fluid (10ml) comprising (g/l 2,4 dioxan):- 2,5 diphenyl oxazole (PPO), 4; napthalene 60; methanol 100ml; ethylene glycol 20ml. Radioactivity in whole dried cells was determined in this scintillation fluid without prior digestion of the sample, as no loss in counting efficiency was observed. Non-aqueous samples were counted in a scintillation fluid (10ml) containing (g/l toluene):- PPO, 25; napthalene 250.

Preparation of O_2 free N_2

O_2 free N_2 was prepared by passage of commercial O_2 free N_2 through a dreschel bottle containing 100ml Fieser's Reagent, which consisted of (g/100ml):- KOH, 20g; sodium anthraquinone-2-sulphonic acid, 2g; and sodium dithionite, 15g.

Traces of dithionite and KOH were removed by passage through two dreschel bottles containing distilled water. Exhaustion of the reagent was indicated by a colour change to dull red, or by the appearance of a precipitate.

Instruments

All centrifugation was performed using a Sorvall superspeed RC-2B refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.), unless otherwise stated. Radioactivity was measured using a Beckman LS-233 liquid scintillation spectrometer (Beckman Instruments Ltd., Glenrothes, Scotland). Spectrophotometric determinations were made using SP600 or SP1800 spectrophotometers (Pye-Unicam Instruments, Cambridge, England), or a Cary 14u.v. recording spectrophotometer (Cary Instruments, Monrovia, California, U.S.A.). If not stated, an SP600 spectrophotmeter was the instrument used.

The Davis Cathode Ray Polarograph was from Southern Analytical Ltd., Camberley, Surrey, England.

Ultrafiltration was carried out using an Amicon stainless steel reservoir RS4 plus concentration-dialysis selector CDS10, with an Amicon Ultrafiltration Cell model 52 (Amicon Corp., Lexington, Mass.,U.S.A.). Evaporation under reduced pressure was performed using a Büchi Rotavapor R. (Büchi Scientific Apparatus, Flarvil, Switzerland).

Source of Chemicals

All chemicals used were of the analytical reagent grade wherever possible, and were obtained from Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire, England or B.D.H. Chemicals Ltd., Poole, Dorset, England.

Tween 80, sodium pyruvate, deoxyribonuclease II, NADH, bovine serum albumin fraction V, PAS, C_3P, antimycin A, Triton 101, lysozyme and P M S were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England.

Alumina ("for chromatography" grade) was obtained from May & Baker Ltd., Dagenham, Essex, England.

Potassium cyanide, DCPIP and sodium malate were obtained from Hopkin & Williams, Chadwell Heath, Essex, England.

$ZnSO_4.7H_2O$, $MnSO_4.5H_2O$, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ ("specpure" grade) were obtained from Johnson and Matthey Chemicals Ltd., London, England.

RESULTS

Chapter 1 Ferrimycobactin Reductase

The purpose of the experiments carried out in this chapter was to continue the study of the enzyme ferrimycobactin reductase, first detected and assayed in our laboratory by Ratledge (1971) and then by Brown & Ratledge (1975) (see Introduction). The experiments, described in detail by Brown (1975) developed an assay system for the enzyme and research was now aimed at characterising the enzyme in order to understand more about its action *in vivo*.

Initial attempts to assay this enzyme were unsuccessful as the activities obtained were unreproducible and were not linear with respect to protein concentration. In addition, the low activity of the enzyme enhanced the difficulties of obtaining a reproducible system on which to base further experiments. Consequently, the primary task was to establish a reproducible activity before proceeding.

A. Concentration and Purification of Ferrimycobactin Reductase

(i) Concentration Due to the low activity obtained using cell free extracts of *M. smegmatis*, concentration of ferrimycobactin reductase by use of the 'Amicon' Ultrafiltration unit was attempted (as in Materials and Methods). By this method, it was hoped that the concentrated enzyme would allow faster rates to be measured hence eliminating some of the difficulties associated with the assay.

Unfortunately this method inactivated the enzyme;

TABLE 1

Partial Purification of Ferrimycobactin Reductase

Purification Step	Protein Concentration (mg/ml)	Total Protein (mg)	Specific Activity (nmol $min^{-1}mg$ $protein^{-1}$)	Total Activity (units)
1. None	9.60	663	0.28	194
2. Streptomycin Sulphate 0-1% supernatant	6.92	530	0.30	159
3. Streptomycin Sulphate 1-2% Supernatant	6.71	503	0.32	158
4. Ammonium Sulphate 0-2% Pellet	0.88	10	0	0
5. Ammonium Sulphate 20-40% Pellet	9.99	120	0.48	57
Ammonium Sulphate 40% Supernatant	1.83	350	0	0

Legend:- Ferrimycobactin reductase activity was purified using streptomycin sulphate followed by ammonium sulphate, at the concentrations shown in the table. Protein concentration and enzyme activity were determined as in Materials and Methods.
1 unit of activity = 1nmol ferrimycobactin reduced $min^{-1}mg$ $protein^{-1}$.

no activity could be detected in either the dialysate or in the material that was washed off the filter. Recombination of the dialysate and filtered material did not restore activity. Repeating the procedure incorporating 1% dithiothreitol (w/v) in the buffer used during the production of the cell free extract did not prevent loss of activity.

(ii) Purification Ferrimycobactin reductase activity was partially purified using streptomycin sulphate followed by ammonium sulphate, as shown in Table 1. No activity was detected except in the material precipitated between 20% and 40% saturation of ammonium sulphate. However over 60% of the total activity was lost on carring out this ammonium sulphate step. No increase in activity was observed on recombining these ammonium sulphate fractions.

Further purification by chromatography through a Sepharose 6B column was attempted using the material precipitated between 20% and 40% ammonium sulphate saturation. This resulted in complete inactivation of the enzyme, and recombination of all the protein-containing fractions eluted from the column did not restore activity.

For future experiments it was decided to employ the supernatant from the 0-2% streptomycin sulphate step, as assays using this material were now reproducible and moreover linear with respect to protein concentration. The onset of reproducibility with streptomycin sulphate purification, may have been related to the reduction in the turbidity of the extract that occurred on carrying out this step. Turbidity of a solution is known to cause light scattering and it is possible that this effect was responsible for the non-linearity observed when

non-purified extracts were used.

B Studies on Ferrimycobactin Reductase

(i) Reduction of Ferrimycobactin by Ferrimycobactin Reductase

As some organisms, e.g. *E. coli*, possess enzymes which hydrolyse their iron chelates, it was necessary to ascertain that the activity observed when assaying for ferrimycobactin reductase was due to reduction and not hydrolysis of the ferrimycobactin. Ferrimycobactin was reduced enzymically in the usual manner, employing a freshly prepared cell-free extract of iron-deficient *M. smegmatis*. Ferrimycobactin was also reduced chemically by using dithionite. This was performed with and without an inactive protein, bovine serum albumin being present to determine whether the presence of protein would interfere with the extraction of mycobactin. After both chemical and enzymic reduction, desferrimycobactin was recovered from the assay mixture as shown in Table 2.

After reduction by dithionite (both in the presence and absence of bovine serum albumin), greater than 90% of the ferrimycobactin reduced was recovered from the assay mixture (Table 2). When reduced enzymically, 90% of the ferrimycobactin could be recovered, which was regarded as evidence of reduction, rather than hydrolysis of ferrimycobactin, being the means of releasing iron from the chelate.

(ii) Activity of Ferrimycobactin Reductase

The activity of this enzyme was measured under the usual conditions (see Materials and Methods) with independent omission of each of the constituents of the assay. Ferrimycobactin, NADH, EDTA and anaerobicity were

TABLE 2

Reduction of Ferrimycobactin By Ferrimycobactin Reductase

Method of Reduction	% Ferrimycobactin Recovered
Dithionite[a]	95%
Dithionite plus bovine serum albumin[b]	92%
Enzymic[c]	90%

Legend:- Ferrimycobactin (400nmoles) was reduced under the usual assay conditions (see Materials and Methods) by:

[a]Dithionite (1.0ml of a freshly prepared sodium dithionite solution, (10mg/ml, w/v)

[b]Dithionite (as in[a]) plus B.S.A. (1.0ml of a 10mg/ml solution (w/v)

[c]Enzymically, using a cell free extract prepared from *M. smegmatis* (1.0ml of a cell free extract, protein concentration ≃ 10mg/ml).
On complete reduction of the ferrimycobactin, ferric chloride was added, and the mycobactin extracted into chloroform as the ferric complex. The volume was decreased by rotary evaporation under reduced pressure, and the mycobactin estimated in the usual manner (see Materials and Methods).

all required for activity of this enzyme; no activity was seen in their absence. The variation of activity with changing NADH and ferrimycobactin concentrations is examined later in this chapter.

Activity of this enzyme was lost on boiling the cell free extract for 5 minutes. This was regarded as evidence of the protein-based nature of this activity. The activity was stable however for up to five days at $2^{o}C$, with no loss of activity over this period.

(iii) Determination of the K_m for NADH and Ferrimycobactin

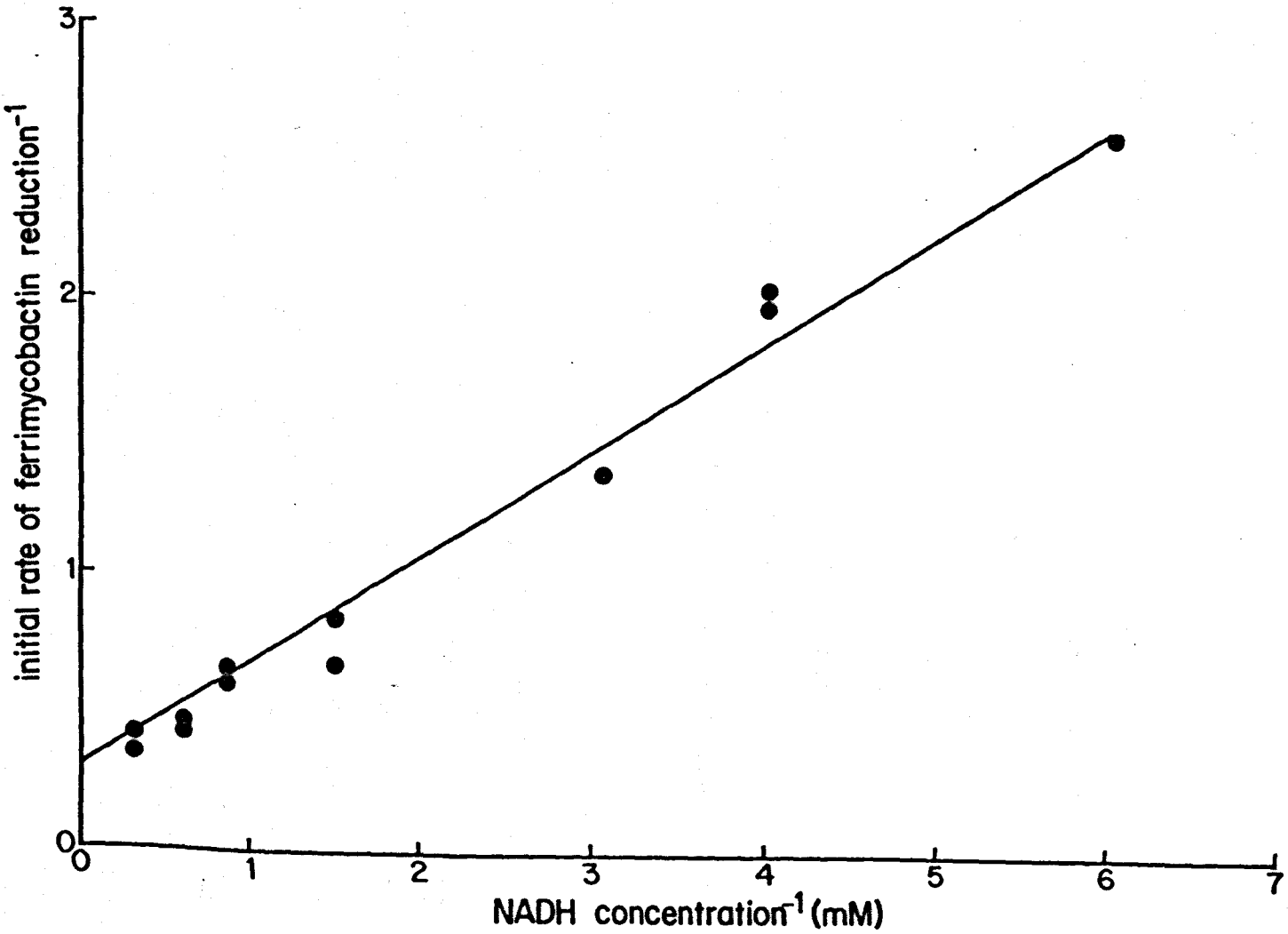
The K_m of ferrimycobactin reductase for NADH was determined over a NADH concentration range of 0.166m$\underline{M}$ to 3.33m$\underline{M}$, while the concentration of ferrimycobactin was maintained at 133.3$\mu\underline{M}$. From Figure 4, the double reciprocal plot of initial rate of ferrimycobactin reduction against NADH concentration, the K_m for NADH was determined as 1.75m$\underline{M}$, with a V_{max} of 3.3nmoles ferrimycobactin reduced $min^{-1}mg\ protein^{-1}$.

The K_m of ferrimycobactin reductase for ferrimycobactin could not be determined. A ferrimycobactin concentration range of 133.3$\mu\underline{M}$ to 8.3$\mu\underline{M}$ was used (keeping the Triton 101 concentration constant), with no significant decrease in initial rate observed over this concentration range. Ferrimycobactin concentration could not be decreased below 8.3$\mu\underline{M}$ because the absorbance change at 450nm that would accompany the reduction of all the ferrimycobactin present would be less than 0.03. This was too small to be monitored spectrophotometrically. Thus the value of the K_m for ferrimycobactin must be 4$\mu\underline{M}$ or less.

Legend to Figure 4: Ferrimycobactin reductase activity was assayed as in Materials and Methods. NADH concentration was varied from 0.166 to 3.33mM. Ferrimycobactin concentration was kept at 133.3μM. From the figure K_m for NADH = 1.75mM and V_{max} = 3.33nmoles ferrimycobactim reduced min^{-1} $mg\ protein^{-1}$.

Initial rate of ferrimycobactin reduction is measured in nmoles ferrimycobactin reduced min^{-1}.

Figure 4. Double Reciprocal Plot Of Initial Rate Of Ferrimycobactin Reduction Against NADH Concentration.



(iv) Determination of the Redox Potential of Ferrimycobactin

In order to determine the redox potential (E_o') of ferrimycobactin, a calibration graph was constructed as described earlier (see Materials and Methods). The iron-containing compounds employed for this purpose are listed in the legend to Figure 5. The reduction voltage of ferrimycobactin (and several other compounds) was determined polarographically, from which the values for the E_o' were obtained using the calibration graph. The compounds examined and their E_o' values obtained are listed in Table 3. Using the value obtained for the E_o' of ferrimycobactin, the equilibrium constant could be calculated for the reaction:-

$$Fe^{3+} \text{ mycobactin} + e^- \rightleftharpoons Fe^{2+} \text{ mycobactin}$$

A value of 5.5×10^{-5} was obtained for ferrimycobactin S, indicating that the equilibrium was very strongly in favour of the oxidised (Fe^{3+}) state. However, the equilibrium constant with NADH as the electron donor would be 8.2, which is weakly in favour of the reduced state.

(v) Effects of Inhibitors on Ferrimycobactin Reductase

The effects of a variety of inhibitors on the activity of ferrimycobactin reductase were examined. Most potent were the sulphydryl reagents NEM and $HgCl_2$ at 0.1mM, producing 39% and 45% inhibition respectively. Activity was weakly inhibited by the uncouplers tested, CCCP and DNP, while KCN at 10mM (an electron transport inhibitor) strongly inhibited activity (Table 4). Data is also presented for comparison on the effects of these inhibitors on iron transport from ferri-exochelin in *M. smegmatis* (Table 4), which is discussed later (see Discussion).

(vi) Unloading of Iron from Ferrimycobactin in Iron-Deficient *M. smegmatis*

In order to observe the action of ferrimycobactin reductase

TABLE 3

The Redox Potential of Ferrimycobactin And Related Compounds

Compound[a] Examined	Reduction Voltage (v) (by polarography)	E_o' (v) (from calibration graph)
Ferrimycobactin S	0.78	-0.265
Ferrimycobactin P	0.77	-0.270
Ferrimycobactin M	0.77	-0.270
Ferrinocobactin 318	0.66	-0.325
Ferrinocobactin 231	0.69	-0.295
Ferrinocobactin 14	0.69	-0.295
Ferriexochelin MS (3A)	0.68	-0.305

Legend:- The reduction voltages of the above compounds were determined polarographically as described (see Materials and Methods) and the E_o' found from the calibration graph (Figure 5).

[a]All the compounds were used at a final concentration of 0.03%.

Legend to Figure 5:- The reduction voltages of certain iron-containing compounds of known E_o' were determined polarographically as described in Materials and Methods.

Compounds used:-		E_o' (mv)
1. Ferric acetate	pH5.0	+340
2. Ferric salicylate	pH4.0	+260
3. Cytochrome c	pH7.0	+254
4. Haemoglobin	pH6.0	+170
5. Haemoglobin	pH7.0	+144
6. Myoglobin	pH7.0	+ 46
7. Ferric oxalate	pH7.0	+ 2
8. Ferric pyrophosphate	pH7.0	-140
9. Peroxidase	pH7.0	-271

E_o' values obtained from the 'Handbook Of Biochemistry', 2nd edition (ed. Sober, H.A.) Chemical Rubber Co.

All compounds used were at a final concentration of 1mM.

Figure 5. Calibration Graphy Of Electrode Potential Against Reduction Voltage By Differential Cathode Ray Polarography.

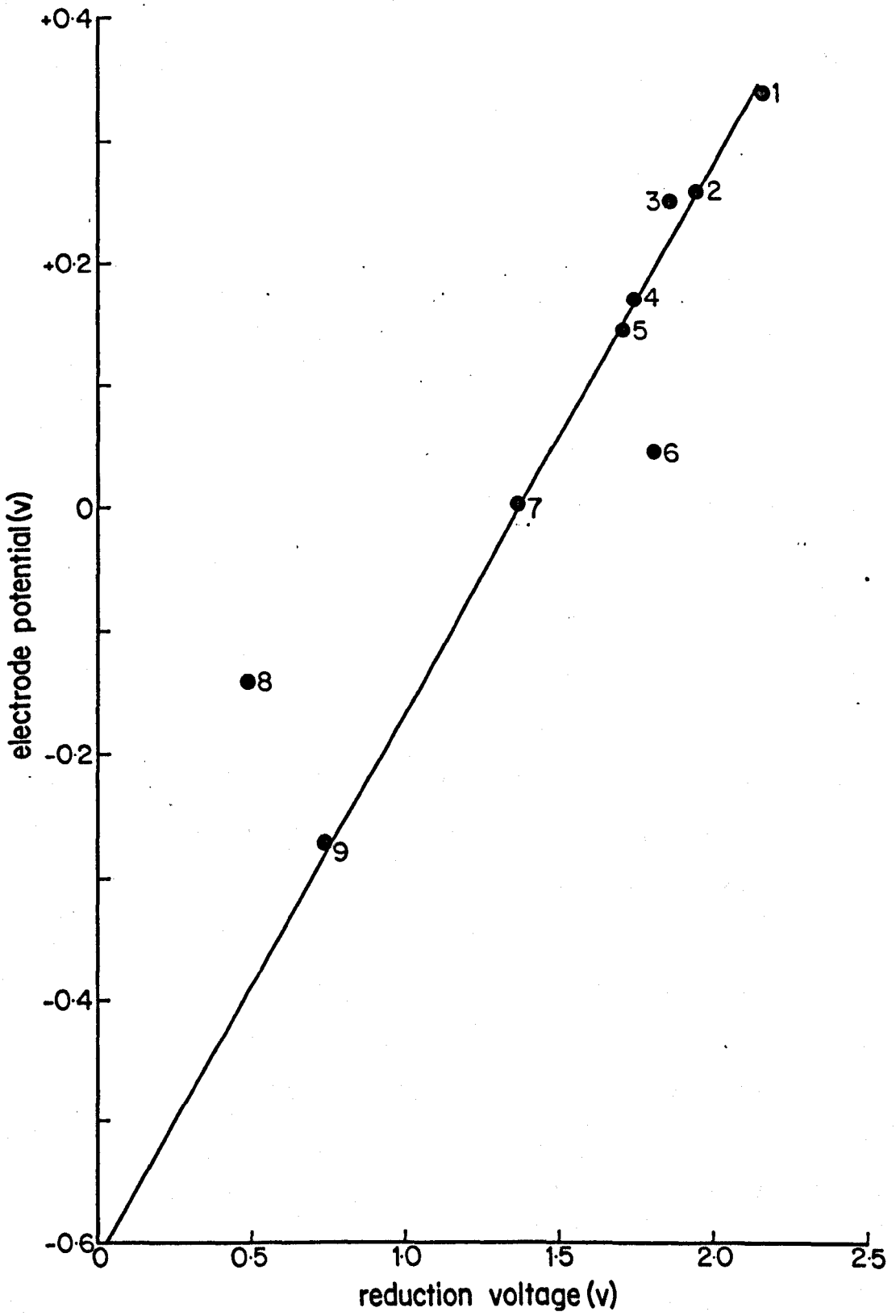


TABLE 4

Relative Effect Of Inhibitors On Ferrimycobactin Reductase

Inhibitor	Final Concentration (mM)	% Inhibition Of Ferrimycobactin Reductase[a]	% Inhibition Of Iron Transport from Ferri-exochelin[b]
0	-	0	0
KCN	1	11	71
	10	74	96
NaN_3	5	13	69
	30	31	97
CCCP	0.01	0	51
	0.10	17	97
DNP	2.0	30	97
NEM	0.1	39	49
$HgCl_2$	0.1	45	96

Legend:- Ferrimycobactin reductase activity was determined as described in Materials and Methods. For CCCP and DNP, controls with the appropriate amount of methanol included were used.

[a]Enzyme activity in the absence of any inhibitor was 0.2nmol min^{-1} mg $protein^{-1}$.

[b]Data obtained from Stephenson and Ratledge (1979). Iron was presented to iron-deficient cells as ferri-exochelin (1.3μMFe) and transport of iron monitored as described in the above communication. Iron uptake in the absence of inhibitor = 37-72nmol Fe min^{-1} g dry $weight^{-1}$.

in vivo, the intracellular release of iron from ferrimycobactin was followed in iron-deficient cells. Ferrimycobactin was generated by the addition of 'soluble' iron to the growth medium, to increase the extracellular iron concentration from 0.05µg/ml to 2.0µg/ml, in the same manner as employed in the 'shift-up' experiments (Results, Chapter 2).

Iron was released from the ferrimycobactin at the rate shown in Figure 6, with 40% of the iron initially associated with the mycobactin being released into the cell over a 12 hour period. (With 200µg iron added per culture flask, this is a loss of iron from ferrimycobactin at 1.6µmoles Fe lost/12h per flask; each 3 day culture of iron-deficient *M. smegmatis* has a dry weight of approximately 300mg, of which 45-50mg protein are released when preparing a cell-free extract). This gives a rate for the loss of iron from ferrimycobactin *in situ* as approximately 3nmoles Fe h^{-1}mg $protein^{-1}$.

This is to be compared to a rate obtained from the *in vitro* spectrophotometric assay of 12nmoles Fe lost h^{-1}mg $protein^{-1}$.

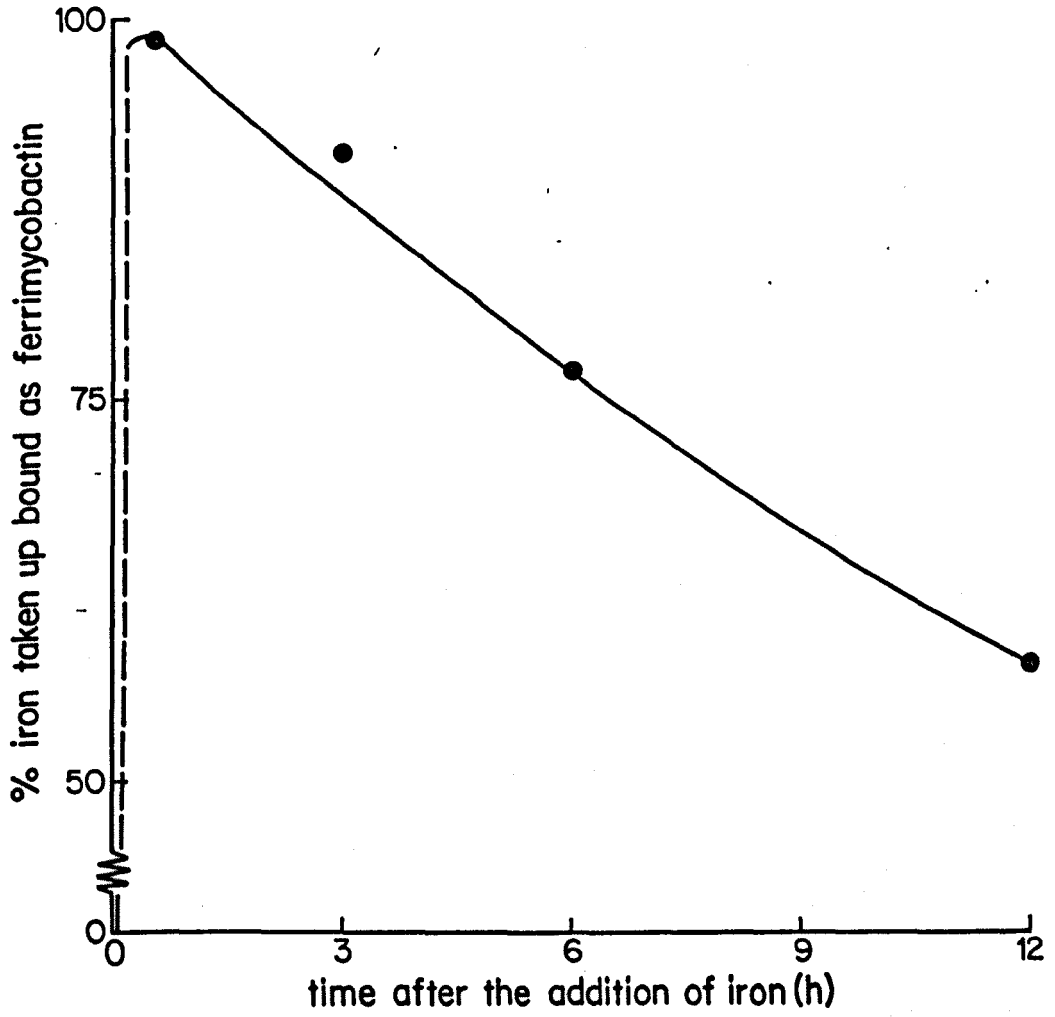
(vii) Preparation of Electron Transport Particles from *M. smegmatis*

Electron transport particles (ETP) were prepared from *M. smegmatis* in order to attempt to study ferrimycobactin reductase in a membrane bound situation. This should be close to the environment of the enzyme *in vivo*, if its function is to reduce ferrimycobactin which itself is probably membrane associated.

Each ETP preparation was assayed for succinate dehydrogenase activity in order to ascertain that the preparations were active; this activity was lost when the preparations were disrupted in any way. The specific activity of this enzyme varied with the preparation, between values of 200 and 400nmol DCPIP reduced

Legend to Figure 6:- Iron was added to iron-deficient *M. smegmatis* as described in Materials and Methods. Iron bound to mycobactin was determined by sonication of the cell suspension (5min at maximum output using the Dawe Soniprobe) in the presence of EDTA at a final concentration of 100mM, prior to the extraction of ferrimycobactin into ethanol. Extraction was performed using iron-free glassware at all times, and 'ANLAR' grade chemicals.

Figure 6. Unloading Of Iron From Ferrimycobactin In Iron-Deficient *M. smegmatis*.



min^{-1} mg $protein^{-1}$. However ferrimycobactin reductase activity could not be detected in every active ETP preparation When present, the activity varied greatly, up to a maximum of 0.8nmoles ferrimycobactin reduced min^{-1} mg $protein^{-1}$. With ETP preparations that lacked ferrimycobactin reductase activity, the addition of the supernatant fluid (from the high speed centrefugation preparative stage) to the ETP preparation did not promote activity of this enzyme.

(viii) Attempted Preparation of Cell Ghosts from *M. smegmatis*

In conjunction with the preparation of electron transport particles (see above), attempts were made to prepare 'cell ghosts' from *M. smegmatis* by a method applied to a similar organism, *M. phlei*. Unfortunately cell ghosts could not be prepared from *M. smegmatis* as osmotically sensitive cells could not be produced. When the lysozyme treatment (which produces the osmotically sensitive cells) was monitored, no decrease in the turbidity of the water-lysed sample as opposed to the control, was observed. Extending the incubation time with lysozyme to 4 hours had no effect. The incubation period of the growing cells with glycine and sucrose was extended from 3 to 17 hours, but with no effect on the production of osmotically sensitive cells. Increasing the lysozyme concentration 10-fold, and incubating the cells with lysozyme in the presence of EDTA (both at 1m$\underline{M}$ and 10m$\underline{M}$) had no effect.

Checks carried out microscopically after the lysozyme incubation demonstrated that the cells were still rod-shaped and not spherical. As an additional check, sucrose density gradient centrifugation carried out on

lysozyme-treated cells demonstrated that there was very little production of osmotically sensitive cells.

C The Non-Specific Nature of Ferrimycobactin Reductase

(i) Activity In Iron-sufficient Cells

M. smegmatis grown iron-sufficiently and iron-deficiently was examined for the presence of ferrimycobactin reductase activity, as described in the legend to Table 5. Activity was observed in both iron-deficient and iron-sufficient cells; the level of this activity was slightly higher in iron-sufficient cells (0.131nmol min^{-1}mg $protein^{-1}$) compared to iron-deficient cells (0.114nmol min^{-1}mg $protein^{-1}$). Considering the function of this enzyme this result was surprising, as iron-sufficient cells have little or no mycobactin (see Results, Chapter 2). However although an explanation for this has been forwarded (see Introduction), other organisms were examined for the presence of ferrimycobactin reductase activity.

(ii) Activity in Other Organisms

A bacterium and a yeast, *Escherichia coli* and *Candida utilis* were examined for ferrimycobactin reductase activity, to determine whether the enzyme responsible for this activity was specifically synthesised by *Mycobacteria*. Activity was detected in extracts prepared from both of these organisms, at 0.205 and 0.127nmol ferrimycobactin reduced min^{-1}mg $protein^{-1}$ respectively (Table 6). These activities are of the same order of magnitude as that obtained using *M. smegmatis*. Similar activities of ferrimycobactin reductase were obtained with *E coli* and *C. utilis* on repeating this experiment.

TABLE 5

Ferrimycobactin Reductase Activity in Iron-Sufficient And Iron-Deficient *M. smegmatis*

Source of Enzyme	Specific Activity (nmol min^{-1} mg $protein^{-1}$)
Iron-Sufficient Cells	0.134
Iron-Deficient Cells	0.114

Legend: Iron-sufficiently and iron-deficiently grown cells were used for the preparation of a cell free extract, and assay of ferrimycobactin reductase, as previously described (see Materials and Methods). Both cultures were grown from the same inoculum).

TABLE 6

Ferrimycobactin Reductase Activity
In Other Organisms

Source of Enzyme	Specific Activity (nmol $min^{-1}mg$ $protein^{-1}$)
E coli	0.205
C. utilis	0.127

Legend:- *C. utilis* and *E. coli* were grown in the same manner as *M. smegmatis*, under iron sufficient conditions, and cell free extracts were prepared and assayed for the presence of ferrimycobactin reductase activity as described earlier (Materials and Methods).

(iii) Reduction of Ferrimycobactin Involving Thiol Groups

The apparent non-specific nature of ferrimycobactin reductase as seen above, prompted the search for possible mechanisms for reduction of ferrimycobactin that could account for the observed activity. Experiments were carried out in the absence of protein, incorporating into the assay materials that contained reduced thiol groups. Cysteine and dithiothreitol both reduced ferrimycobactin when incorporated into the usual assay (i.e. plus EDTA) at final concentrations of 33.3mM and 3.33mM. Mercaptoethanol incorporated at 33.3mM also reduced ferrimycobactin (Table 7). Also tested was alcohol dehydrogenase, which is known to contain 25-30 reduced thiol groups per molecule. This protein also reduced ferrimycobactin under these conditions.

Incubation of all the thiol-containing reagents with iodoacetamide overnight (at a final concentration of 10μM) prior to the assay prevented the reduction of ferrimycobactin. Iodoacetamide binds irreversably to thiol groups, thus implicating their involvement in the above experiments. Overnight incubation of cell free extracts prepared from *M. smegmatis* with 10μM iodoacetamide caused a 50 - 60% inhibition in the activity of ferrimycobactin reductase. No further inhibition was detected on increasing the iodoacetamide concentration to 10mM. Similar results were obtained using cell free extracts prepared from *E. coli* and *C. utilis*, which also exhibited 50-60% inhibition of activity after incubation with 10μM iodoacetamide. Again, no further inhibition was observed on increasing the iodoacetamide concentration to 10mM.

TABLE 7

Ferrimycobactin Reductase Activity Involving Thiol Groups

Thiol-Containing Reagent	Final Concentration (mM)	Activity (nmol ferrimycobactin reduced min^{-1})
Cysteine	3.33	1.70
Dithiothreitol	3.33	0.89
	33.3	1.70
Mercaptoethanol	3.33	0
	33.3	0.89
Yeast alcohol dehydrogenase	3.3mg/ml	3.7

Legend:- Ferrimycobactin reduction was assayed as described in Materials and Methods, only omitting the enzyme and substituting one of the above thiol-containing reagents. The activity observed using a cell free extract prepared from *M. smegmatis* (1.0ml of an extract, of protein concentration 10mg/ml) was 2-4nmol ferrimycobactin reduced min^{-1}.

(iv) Activity of Ferrimycobactin Reductase Using Other Electron Donors

Using cell free extracts prepared from *M. smegmatis*, several compounds were tested as possible substitutes for NADH as electron donor in the ferrimycobactin reductase assay. Malate, β-hydroxybutyrate and quinol (all added to give a final concentration of 3.33mM) were incorporated into the assay with catalytic amounts of NAD^+ (100nmoles), but were ineffective as electron donors. Increasing the final concentration of these compounds to 33.3mM and the amount of NAD^+ added to 10μmoles, did not promote ferrimycobactin reductase activity.

(v) Activity of Ferrimycobactin Reductase Using Other Substrates

Several iron-containing compounds were incorporated into the enzyme assay for ferrimycobactin reductase, substituting for ferrimycobactin to determine the specificty of this activity for its substrate. Tested were ferriexochelin MS (the extracellular iron-chelating agent produced by *M. smegmatis)*; ferrioxamine B (the iron-chelating agent produced by *Streptomyces spp.)* and ferric ammonium citrate.

All three compounds assayed could be reduced under anaerobic conditions using cell free extracts of *M. smegmatis*, Ferrioxamine B being the most rapidly reduced, at 0.34nmol min^{-1} mg $protein^{-1}$. Ferriexochelin MS and ferric ammonium citrate were also reduced, at the slower rates of 0.14 and 0.16nmol min^{-1}mg $protein^{-1}$ respectively (Table 8). In this experiment, ferrimycobactin reductase was assayed using ferrimycobactin as the substrate, under the same

TABLE 8

Assay Of Ferrimycobactin Reductase Activity Using Other Substrates

Substrate	Activity (nmol reduced min^{-1} mg $protein^{-1}$)
Ferriexochelin MS	0.14
Ferrioxamine B	0.34
Ferric Ammonium Citrate	0.16
Ferrimycobactin	0.38

Legend:- Assays were performed as in the Materials and Methods, except that EDTA was replaced by salicylic acid (at a final concentration of 0.12mM) as a trap for ferrous ions. Salicylic acid was added to the anaerobic cuvette from the side arm after gassing with O_2-free N_2. The reaction was initiated by the addition of NADH from an anaerobic vessel using a pre-gassed syringe. The reaction was monitored by recording the change in absorbance at 450nm, except for ferriexochelin, which was monitored at 430nm.

The activity observed using EDTA and ferrimycobactin in the assay was 0.3 - 0.4nmol reduced min^{-1} mg $protein^{-1}$. With substrates, no activity was seen in the absence of enzyme, or before the addition of NADH.

conditions as a control (Table 8). It is of interest to note that salicylate can substitute for EDTA as an acceptor of ferrous ions, as this may have some physiological significance. This is discussed fully in the Discussion.

Chapter 2 Growth And Metabolite Concentrations Of *Mycobacterium smegmatis* During Iron-Sufficient and Iron-Deficient Growth

Concentrations Of Metabolites

M. smegmatis grown iron-deficiently and iron-sufficiently, was examined to ascertain the effects of iron on growth and certain metabolites. Cell growth was less during iron-deficiency compared to iron-sufficient conditions, while the intracellular concentrations of total iron and non-haem iron were also lower (Table 9). Non-haem iron constituted about 85% of the total iron in iron-sufficiently grown cells after 3 days' growth, but this proportion fell to about 65% with further growth up to 7 days. The same appeared to be true of iron deficiently grown cells although the non-haem iron, as a proportion of the total iron, declined even faster with growth. The amounts of total iron in iron-deficiently grown *M. smegmatis* showed that, at this extracellular iron concentration of 0.05µg/ml, the organism could scavenge all the available iron from the growth medium; however during iron-sufficient growth with 2.0µg Fe/ml the organism could acquire 75-80% of the available iron from the growth medium.

The mycobactin concentration rose with the age of the cells during iron-deficient growth, as expected (Table 9). Mycobactin was not detected in iron-sufficiently grown cells, which means that under the experimental conditions employed mycobactin may only be present at a concentration of 50µg/g dry weight or less.

In contrast to this, cytochrome concentrations were not greatly affected by the iron status of the organism (Table 10).

TABLE 9

Levels Of Iron, Mycobactin and Cell Dry Weight In *M. smegmatis* Grown Iron-Deficiently And Iron-Sufficiently

Growth (days)	Cell Dry Weight (g/100ml)	Intracellular Iron Total Iron (µmol/g dry wt)	Intracellular Iron Non-Haem Iron (µmol/g dry wt)	Mycobactin Concentration (mg/gdry wt)
a) Iron-Sufficient Growth				
3	0.347	7.9	6.7	n.d.
4	0.485	6.2	4.8	n.d.
5	0.499	5.6	3.9	n.d.
7	0.605	5.0	3.4	n.d.
b) Iron-Deficient Growth				
3	0.295	0.32	0.27	13.9
4	0.341	0.29	0.18	25.8
5	0.362	0.20	0.12	28.1

Legend:- *M. smegmatis* was grown iron-deficiently and iron sufficiently for 3 - 7 days, and samples taken for the determination of cell dry wt., total iron, non-haem iron and mycobactin concentration (as in Materials and Methods).

n.d. = not detectable (below 50µg/g dry wt)

TABLE 10

Flavoprotein And Cytochrome Concentrations In *M. smegmatis* Grown Iron-Deficiently And Iron-Sufficiently

Growth (days)	Flavoprotein	Cytochromes			Total Flavoprotein and Cytochrome Concentration (nmol/μmol cell Fe)
		c	b	a	
	(nmol/g dry wt)				
a) Iron-Sufficient Growth					
3	20.2	4.3	n.d.	n.d.	3.1
4	26.1	6.4	1.7	1.4	5.7
5	33.5	8.9	4.4	5.9	9.4
7	71.2	12.7	7.4	12.2	20.7
b) Iron-Deficient Growth					
3	20.5	9.2	n.d.	n.d.	92.8
4	21.1	9.7	n.d.	n.d.	106.2
5.	24.2	6.1	1.2	1.7	166.0

Legend:- *M. smegmatis* was grown iron-sufficiently and iron-deficiently for 3-7 days, and samples taken for determination of flavoprotein and cytochrome concentrations (as in Materials and Methods).
n.d. = not detectable.

Although there was a forty-fold difference in iron concentration between the iron-deficient and iron-sufficient conditions, the concentration of cytochromes was not affected to this extent. Flavoprotein and cytochrome c appeared to be the least affected by iron-deficiency, displaying only a 2-to 3-fold ratio of concentrations between the two iron states. Cytochromes b and a however, showed a greater dependance on the iron status of the cell, with a 5-to 7-fold ratio of concentrations between iron-sufficient and iron-deficient conditions after 5 days' growth. Certainly the overall concentration of cytochromes was maintained during iron-deficiency, which could only have been at the expense of other intracellular iron- containing compounds being depleted (see Discussion).

The levels of porphyrin, both intracellular and extracellular, were also examined during iron-deficient and iron-sufficient growth (Table 11). Both intracellular and extracellular porphyrin concentrations were considerably higher during iron-sufficient growth, especially in the later stages. During iron-sufficient growth, both the cells and the spent growth medium turned pale brown with age, due to the production of porphyrin. Extracellular porphyrin was identified as coproporphyrin III tetra methyl ester, by both spectrophotmetry and paper chromatography (see Materials and Methods). The absorption spectrum obtained using extracellular porphyrin had absorption peaks at 400, 498, 532, 566, 594 and 621nm whose relative peak heights were 121.6, 9.7, 6.7, 4.8, 1.0 and 3.4 respectively. This was in agreement with the spectrum previously recorded for coproporphyrin III tetra methyl ester (Falk, 1964). On paper

TABLE 11

Porphyrin Concentrations and δ-Aminolevulinic Acid Dehydratase Activity In *M. smegmatis* Grown Iron-Deficiently And Iron-Sufficiently

Growth (days)	δ-Aminolevulinic Acid Dehydratase Activity (nmol/30min/mg protein)	Porphyrin Concentrations Intracellular (μmol/g dry wt)	Extracellular (μg/l)
a) Iron-Sufficient Growth			
3	36.4	41.9	1.0
4	18.6	58.3	12.3
5	19.5	123.0	32.5
7	23.6	222.2	112.8
b) Iron-Deficient Growth			
3	41.5	<25	0.4
4	21.8	<25	1.9
5	26.6	<25	8.3

Legend:- *M. smegmatis* was grown iron-sufficiently and iron-deficiently for 3-7 days, and samples taken for the determination of δ-aminolevulinic acid dehydratase activity, and the concentration of intracellular and extracellular porphyrin, (as in Materials and Methods).

chromatography, extracellular porphyrin corresponded to genuine coproporphyrin III tetramethyl ester, both having an R_F value of 0.67. Coproporphyrin III tetra methyl ester has also been shown to be the identity of the intracellular porphyrin produced during growth (Brown, 1975), which was confirmed when identifying the extracellular porphyrin produced.

Enzyme Studies

In order to locate the site responsible for the differences observed above in the porphyrin concentrations (Table 11), the activity of enzymes of porphyrin biosynthesis was investigated in cells grown under iron-deficient and iron-sufficient conditions.

No significant differences were observed in the activity of δ-aminolevulinic acid dehydratase during iron-deficient and iron-sufficient growth (Table 11). Presumably this, the second enzyme of porphyrin biosynthesis, cannot be the site responsible for the above differences in porphyrin concentration during the growth conditions employed. Attempts were made to assay the enzyme δ-aminolevulinic acid synthetase, which is the first enzyme of porphyrin and haem synthesis. However using a wide variety of methods (see Materials and Methods), no activity of this enzyme could be detected. Even using extracts prepared from *Propionibacterium shermanii*, where activity of this enzyme was reported to have been detected (Menon and Shemin, 1967), no activity could be found. This was presumably due to the extreme instability of this enzyme, which has been acknowledged by a large number of other workers (see Jacobs, 1974).

'Shift-Up' Experiments

To determine the response of *M. smegmatis* to a rapid change in the extracellular iron concentration, a series of shift-up experiments were carried out. The rates of change of the metabolites observed above, were followed, to determine the efficiency with which extracellular iron was accumulated, and the speed with which this iron was distributed to various iron-containing cellular components. At 60h after inoculation iron was added to the growth medium of iron-deficiently growing cells to bring the iron concentration to that of iron-sufficiency. Iron was added in three forms: 'soluble' (as soluble ferrous sulphate), 'insoluble' (as precipitated ferric phosphate) and ferriexochelin (see Materials and Methods).

Iron uptake by *M. smegmatis* from all three forms of iron presented to the cell, was rapid as judged by the increase in the intracellular total iron concentration (Table 12). By 3 hours after the addition of iron, the intracellular concentration of iron in these experiments corresponded to the total amount of iron added in each case, suggesting that the organism has the ability to acquire all the iron in its environment, when grown iron-deficiently. This was in agreement with the observations made earlier (see 'Levels of Metabolites'). The greater proportion of the added iron in each case, initially entered the non-haem iron fraction, which represented approximately 70% of the total iron accumulated, 3 hours after the addition of iron. This proportion fell with the age of the cells, as was observed earlier (see 'Levels of Metabolites').

TABLE 12

Concentration Of Total Iron And Non-Haem Iron In Shift-Up Experiments

Time After The Addition Of Iron (h)	Mode of Addition Of Iron					
	Soluble		Insoluble		Ferriexochelin	
	Total Iron	Non-haem Iron	Total Iron	Non-Haem Iron	Total Iron	Non-Haem Iron
	(µmol/g dry wt)					
0	0.48	0.30	0.59	0.40	0.50	0.32
3	9.4	6.9	9.5	6.4	7.8	4.8
6	9.1	6.7	9.0	5.2	7.3	4.5
12	8.9	6.7	9.1	4.8	6.7	3.9
18	8.4	6.0	8.7	4.4	6.6	3.9
24	7.8	4.7	7.7	4.1	6.6	3.2
48	7.6	4.2	7.5	3.9	5.4	2.8

Control (no addition of iron)

	Total Iron	Non-haem Iron
12	0.44	0.26
24	0.36	0.22
48	0.32	0.18

Legend:- *M. smegmatis* was grown iron-defiently for 60h. Iron was added to give a final concentration of 2.0µg/ml with soluble and insoluble iron, and 1.4µg/ml with ferriexochelin. Controls,where no addition of iron was made, were carried out.

Cell growth was increased in iron-deficient cells that received extra iron when compared to iron-deficient cells of equal age. The stimulation observed appeared to be the same for all three forms of addition of iron (Figure 7). The concentration of mycobactin, as expected, declined on the addition of iron, while its concentration continued to rise in iron-deficient cells (Figure 8). The decrease in mycobactin concentration was most rapid when iron was added as soluble iron. With iron added as insoluble iron or as ferriexochelin the concentration of mycobactin continued to rise for 3-6 hours after the iron had been added to the growing cells. This was not seen when iron was added as soluble iron. The decline in mycobactin concentration on the addition of iron (in any of the forms used) appeared to be mainly due to the dilution of the mycobactin as the cell dry weight increased. Very little degradation of mycobactin appeared to have occurred after the iron was added to the cells (Table 13).

The cytochrome levels of *M. smegmatis* when shifted-up from iron-deficiency to iron-sufficiency, followed the trend that was observed earlier for the cytochromes (see Levels of Metabolites). The concentrations of flavoprotein and cytochrome c were least affected by the change in iron status of the cell; slight differences in the concentrations of flavoprotein and cytochrome c were observed between the two iron concentrations used (Tables, 14, 15 and 16). Cytochromes b and a however, were affected to a much greater extent by the addition of iron. A 4-fold concentration difference was seen for

TABLE 13

Total Mycobactin Recovered In Shift-up Experiments

Time After the Addition of Iron (h)	Mode of Addition of Iron		
	Soluble	Insoluble	Ferriexochelin
	(mg/g dry wt)		
0	21.5	21.6	8.7
3	21.2	26.4	11.2
6	20.1	22.6	11.9
12	19.4	20.3	9.3
18	18.5	22.9	9.5
24	20.0	19.8	8.7
48	19.4	16.2	7.5
Control			
12	26.3	31.6	18.1
24	36.9	32.8	23.5
48	43.5	44.9	29.3

Legend:- As for Table 12

Mycobactin concentration was determined as in Materials and Methods.

Legend to Figure 7:- *M. smegmatis* was grown iron-deficiently for 60h. Iron was added to give a final concentration of 2.0µg/ml with soluble (A) and insoluble iron (B), and 1.4µ g/ml with ferriexochelin (C).

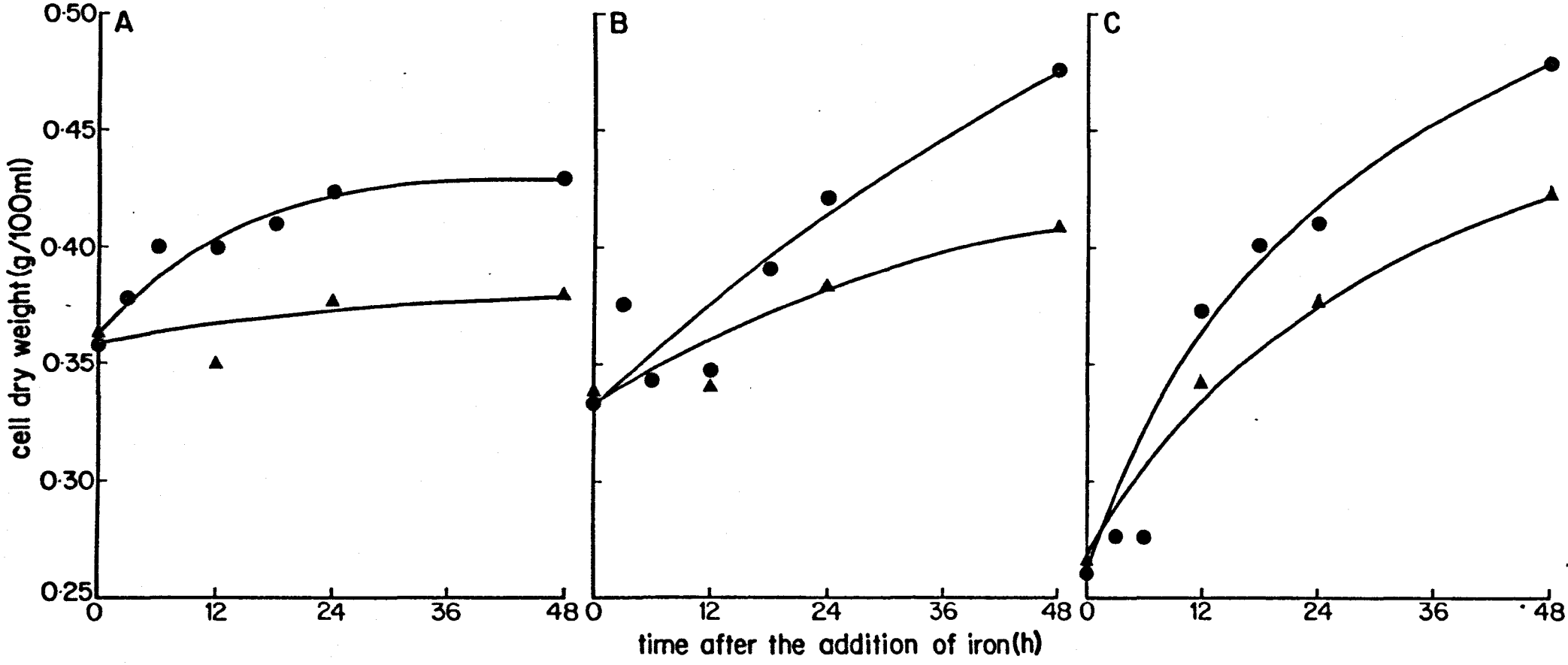
To the control, no addition was made.

Samples were taken after the addition of iron for the determination of the cell dry wt. (as in materials and methods).

O —— O Cell Dry Wt. In shifted-up Cells

Δ —— Δ Cell Dry Wt. In Iron-Deficient Cells (Control).

Figure 7. Changes In Cell Dry Weight On The Addition Of Iron To Iron-Deficiently Grown *M. smegmatis*.



Legend to Figure 8:- As for Figure 7.

Mycobactin concentration was determined as in materials and methods.

O —— O Mycobactin Concentration In Shifted-Up Cells.

Δ —— Δ Mycobactin Concentration in Iron-deficient Cells (Control)

Figure 8 Changes in Mycobactin Concentration On The Addition Of Iron To Iron-Deficiently Grown *M. smegmatis*.

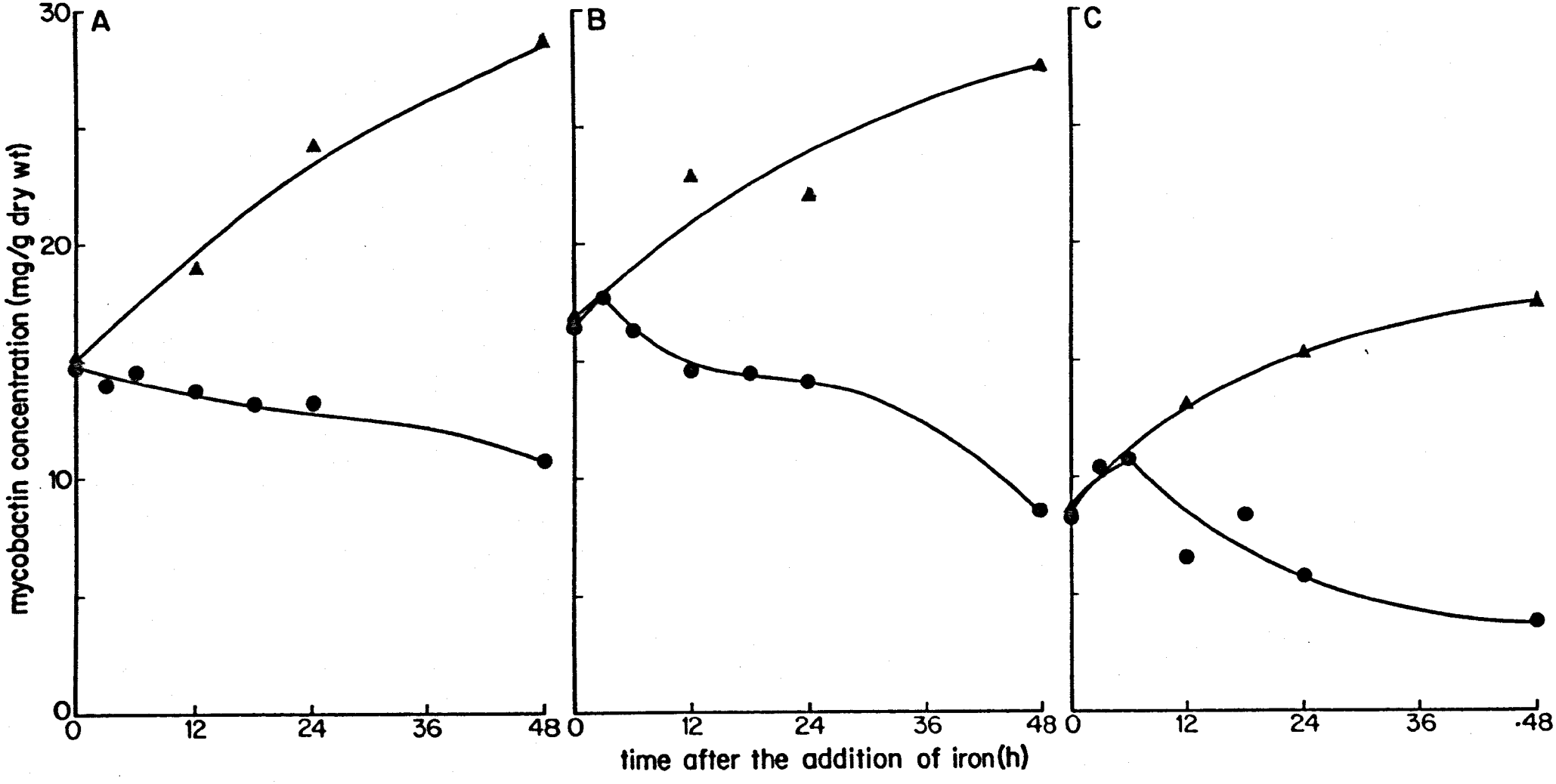


TABLE 14

Concentrations of Flavoprotein And Cytochromes In Shift-up Experiments Using Soluble Iron

Time After The Addition Of Iron (h)	Flavoprotein	Cytochromes		
		c	b	a
	(nmol/g dry wt)			
0	8.2	2.8	n.d.	n.d.
3	9.0	2.9	1.2	n.d.
6	8.5	3.3	2.1	2.9
12	13.1	4.1	2.4	3.9
18	14.3	3.8	3.3	5.6
24	16.1	6.2	4.0	3.7
48	20.2	7.6	6.6	9.1
Control				
12	10.6	4.3	n.d	n.d
24	11.2	5.8	1.5	n.d
48	13.0	7.5	1.8	2.3

Legend:- As for Table 12

Flavoprotein and Cytochrome concentrations were determined as in Materials and Methods.

n.d. = not detectable.

TABLE 15

Concentrations of Flavoprotein and Cytochromes In Shift-Up Experiments Using Insoluble Iron

Time After The Addition of Iron (h)	Flavoprotein	Cytochromes		
		c	b	a
	(nmol/g dry wt)			
0	9.1	1.1	n.d.	n.d.
3	11.7	1.8	1.5	n.d.
6	13.2	3.8	2.5	2.8
12	15.3	3.8	3.3	4.2
18	17.4	5.0	4.4	6.0
24	16.2	6.2	5.4	9.3
48	23.1	7.2	6.1	11.2
Control				
12	11.5	3.3	n.d.	n.d.
24	12.2	4.3	n.d.	n.d.
48	13.6	6.7	1.6	2.5

Legend:- As for Table 12

Flavoprotein and cytochrome concentrations were determined as in Materials and Methods.

n.d. = not detectable.

TABLE 16

Concentrations of Flavoprotein and Cytochromes In Shift-Up Experiments Using Ferriexochelin

Time After The Addition of Iron (h)	Flavoprotein	Cytochromes		
		c	b	a
	(nmol/g dry wt)			
0	11.3	2.2	n.d.	n.d.
3	12.8	2.8	1.6	n.d.
6	13.5	3.4	1.8	1.7
12	15.2	4.8	2.3	3.6
18	15.5	4.2	3.8	5.2
24	16.8	5.7	4.3	6.4
48	18.3	6.3	5.0	7.8
Control				
12	11.6	4.6	n.d.	n.d.
24	12.8	4.9	n.d.	n.d.
48	14.2	5.8	1.2	1.5

Legend:- As for Table 12

Flavoprotein and cytochrome concentrations were determined as in Materials and Methods

n.d. = not detectable.

cytochromes b and a between the iron concentrations employed when the cells were shifted-up to iron-sufficient conditions. As before, the type of added iron made no significant difference to the response of iron-deficient *M. smegmatis* to the increase in the extracellular iron concentration.

The production of intracellular and extracellular porphyrin was affected by the increase in iron concentration. By 48 hours after the addition of iron to the cultures, a 5- to 6- fold increase in the intracellular porphyrin concentration was observed with all three types of added iron (Figure 9). A greater difference was observed in the extracellular porphyrin concentration about an 8- to 10-fold increase when iron was added as soluble and insoluble iron. However a smaller increase, approximately 5- to 6- fold, was observed in the extracellular porphyrin concentration when iron was added as ferriexochelin (Figure 10). The increase in pophyrin production appeared to occur earlier if iron was added as soluble iron, as compared to the increase observed when iron was added as insoluble iron and ferriexochelin.

On the addition of soluble iron to iron-deficient cultures of *M. smegmatis*, the cells were observed to rapidly change colour (over 5 minutes) from creamy-white to bright red. This was attributed to the binding of the added iron by the mycobactin present in the cell wall of the iron-deficient cells. This phenomenon was not seen when iron was added as either insoluble iron or ferriexochelin, or when iron was added to iron-sufficient cells that contained no mycobactin.

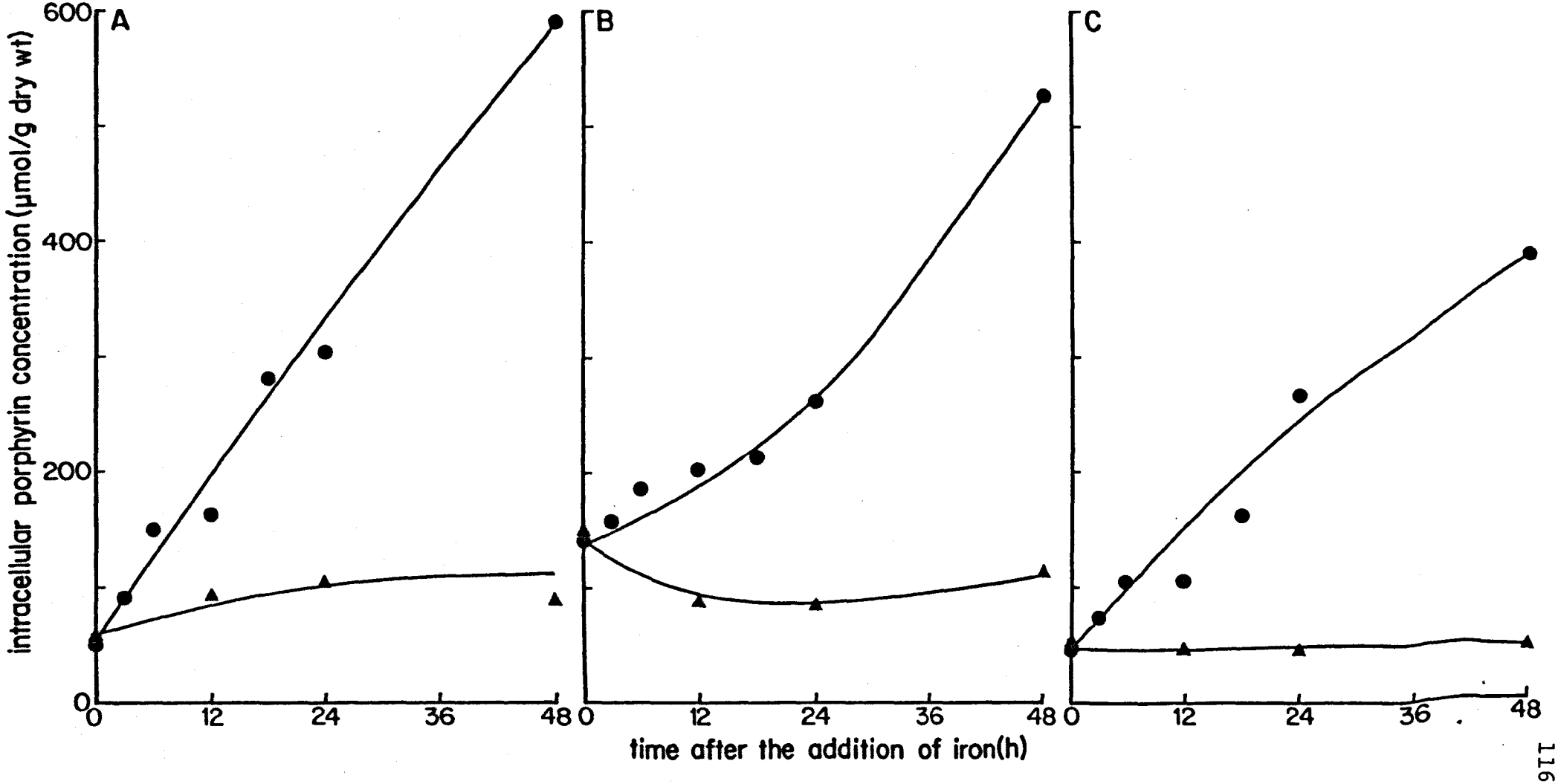
Legend to Figure 9:- As for Figure 7.

Intracelluar porphyrin concentration was determined as in materials and methods.

O —— O Intracellular Porphyrin Concentration In Shifted-Up Cells.

Δ —— Δ Intracellular Porphyrin Concentration In Iron-Deficient Cells (Control)

Figure 9 Changes In Intracellular Porphyrin Concentration On The Addition Of Iron To Iron-Deficiently Grown *M. smegmatis*.



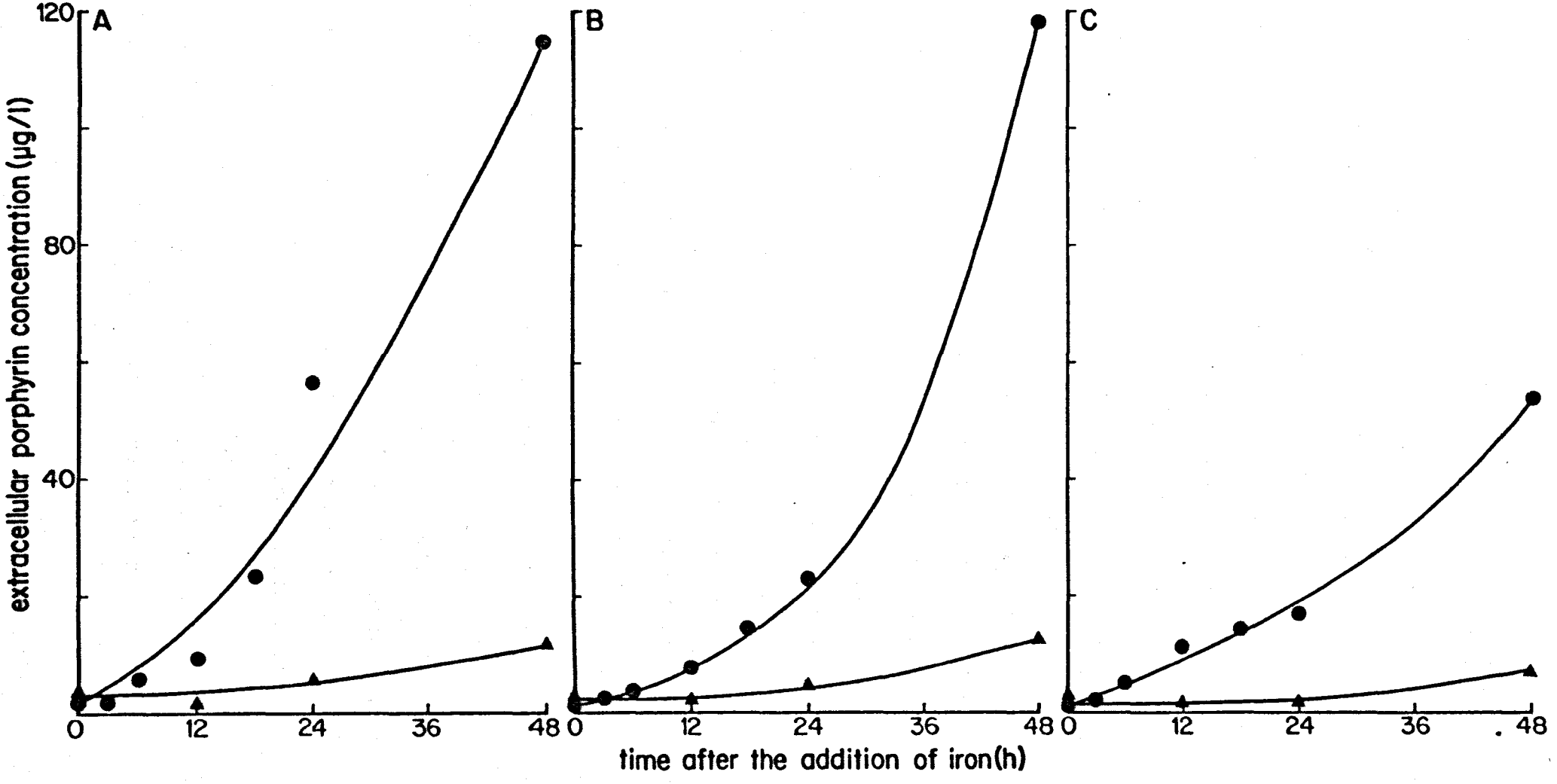
Legend to Figure 10:- As for Figure 7.

Extracellular porphyrin concentration was determined as in Materials and Methods.

o —— o Extracellular porphyrin concentration in Shifted-Up Cells

Δ —— Δ Extracellular porphyrin concentration in Iron-Deficient Cells (Control).

Figure 10. Changes In Extracellular Porphyrin Concentration On The Addition Of Iron To Iron-Deficiently Grown *M. smegmatis*.



Further Shift-Up Experiments Using Higher Extracellular Iron Concentrations

In order to ascertain the effects of iron at concentrations greater than those already used for the growth of *M. smegmatis*, a further series of shift-up experiments were carried out. Iron was added to both iron-deficient and iron-sufficient cultures of *M. smegmatis* (after 60h growth) to give final concentrations from 2.0μgFe/ml to 150.0μgFe/ml i.e. up to 75 times more than that added to give full iron-sufficient growth. The purpose of such experiments was to observe the response of *M. smegmatis* during conditions of iron availability excess to requirements i.e. the condition of "feast" to use the terminology of Koch (see Introduction).

The amount of iron taken up by the cells varied with the iron status of the cell prior to the addition of iron (Tables 17 and 18). Iron-deficient cells had a greater capacity to take up iron, than iron-sufficient cells. An intracellular iron concentration of 150-170μmol/g dry wt. was the maximum concentration attained by iron-deficient cells, (at which level the iron assimilated is 1% of the cell dry weight) and increasing the extracellular iron concentration above 50.0μgFe/ml did not increase this value. In contrast, iron-sufficient cells were able to take up extra iron with each increase in extracellular iron concentration. However the amount of iron accumulated by iron-sufficient cells was lower than that accumulated by iron-deficient cells when equal iron concentrations were presented to the cell.

TABLE 17

Intracellular Iron Concentration Of Iron-Deficiently Grown *M. smegmatis* In Further Shift-up Experiments Using Higher Extracellular Iron Concentrations.

Extracullar Iron Concentration (µg/ml)	1h Intracellular Iron Concentration (µmol/g dry wt)	% Taken up from Growth Medium	24h Intracellular Iron Concentration (µmol/g dry wt)	% Taken Up From Growth Medium
0.05	0.31	100	0.25	100
2.0	10.4	100	9.4	100
25.0	45.9	35	76.5	75
50.0	45.8	18	169.5	82
75.0	59.3	15	133.1	49
100.0	66.2	12	156.4	47
150.0	56.6	7	173.9	33

Legend:- Iron was added to 60h iron-deficient cells to give final iron concentrations from 2.0 to 150 gFe/ml. Samples were taken at 1h and 24h after the addition of iron for the determination of intracellular iron concentration.

TABLE 18

Intracellular Iron Concentration Of Iron-Sufficiently Grown *M. smegmatis* In Further Shift-Up Experiments Using Higher Extracellular Iron Concentrations

Extracellular Iron Concentration (µg/ml)	1h Intracellular Iron Concentration (µmol/g dry wt.)	% Taken Up from Growth Medium	24h Intracellular Iron Concentration (µmol/g dry wt)	% Taken Up From Growth Medium
2.0	8.5	100	7.1	100
25.0	23.8	18	41.3	57
50.0	9.1	4	66.9	45
75.0	7.2	4	74.2	36
100.0	14.5	3	85.6	30
150.0	13.2	2	102.2	25

Legend:- Iron was added to 60h iron-sufficient cells to give final iron concentrations from 25.0 to 150.0µgFe/ml. Samples were taken at 1h and 24h after the addition of iron for the determination of intracellular iron concentration.

In each case, greater than 95% of the iron assimilated was detected in the non-haem iron fraction (this fraction includes any iron bound to mycobactin).

By 24-hours after the addition of iron to iron-deficient cells, the cell dry weight had increased with increasing extracellular iron concentration, compared to iron-deficient cells without added iron (Table 19). However the addition of iron to iron-sufficient cells did not have any effect above an extracellular iron concentration of 25μgFe/ml, compared to those cells without added iron.

Mycobactin concentration, as would be expected, declined after the addition of iron to iron-deficient cells. However when iron concentrationsof 25.0μgFe/ml were employed, the decrease in the concentration of mycobactin was partly due to actual degradation of the mycobactin, as well as dilution, as the cell dry weight increased with time (Table 20). Degradation of mycobactin was not seen when an iron concentration of 2.0μgFe/ml was used, in agreement with the observations made in the previous section (Table 13). No significant differences were observed in the effects of the added iron on mycobactin levels when the iron concentration was increased above 25.0μgFe/ml. It should be noted that, at extracellular iron concentrations of 25.0μgFe/ml and above, enough iron was present to completely saturate all the mycobactin within iron-deficient cells.

The changes observed in the cytochrome concentrations were also partly dependent on the iron status of the cell prior to the addition of iron (Tables 21 and 22). With

TABLE 19

Cell Dry Weight of *M. smegmatis* In Further Shift-Up Experiments Using Higher Extracellular Iron Concentrations

Extracellular Iron Concentration (µg/ml)	Cell Dry Weight (g/100ml) Iron-Deficient Cells 1h	24h	Iron-Sufficient Cells 1h	24h
0.05	0.382	0.401	-	-
2.0	0.318	0.420	0.388	0.532
25.0	0.344	0.439	0.366	0.663
50.0	0.341	0.436	0.432	0.629
75.0	0.350	0.490	0.406	0.663
100.0	0.322	0.538	0.431	0.633
150.0	0.338	0.504	0.333	0.666

Legend:- Iron (as soluble iron) was added to iron-deficient and iron-sufficient cells to give extracellular iron concentrations from 2.0 to 150.0 µg/ml. Samples were taken at 1h and 24h after the addition of iron, for the determination of cell dry weight (as in Materials and Methods).

TABLE 20

Concentration Of Mycobactin In Iron-Deficient *M. smegmatis* In Further Shift-Up Experiments Using Higher Extracellular Iron Concentrations.

Extracellular Iron Concentration (µg/ml)	1h		24h	
	Mycobactin Concentration (mg/g dry wt)	Total Mycobactin Recovered (mg)	Mycobactin Concentration (mg/g dry wt.)	Total Mycobactin Recovered (mg)
0.05	13.51	10.32	16.12	12.92
2.0	12.47	10.42	12.13	10.18
25.0	14.95	10.28	5.75	5.04
50.0	15.01	10.24	8.74	7.62
75.0	16.46	11.52	6.12	5.98
100.0	13.93	11.80	6.28	6.76
150.0	17.40	11.76	6.85	6.90

Legend:- Iron (as soluble iron) was added to iron-deficient cells to give extracellular iron concentrations from 2.0µg/ml to 150.0µg/ml. Samples were taken at 1h and 24h after the addition of iron, for the determination of mycobactin concentration (as in Materials and Methods).

both iron-deficient and iron-sufficient shifted-up cells, increasing the iron concentration above 25.0μgFe/ml made no significant difference to the changes seen. However the final concentration of each of the cytochromes was higher in shifted-up iron-sufficient cells than in the equivalent iron-deficient cells. Cytochrome b especially was affected by the increase in iron concentration, reaching values greater than 30nmol/g dry wt in iron-sufficient cells 24 hours after the addition of iron.

Intracellular porphyrin was maximally produced when iron was added to give an extracellular concentration of 2.0μgFe/ml, with both iron-deficient and iron-sufficient cells (Table 23). However the intracellular porphyrin concentration in shifted-up iron-deficient cells decreased with increasing iron concentration above 2.0μgFe/ml, while in equivalent iron-sufficient cells, the intracellular porphyrin concentration remained constant with increasing iron concentration. In contrast to the cytochrome concentrations (Tables 21 and 22), the highest values for intracellular porphyrin were obtained with shifted-up iron-deficient cells, which had a 2- to 3- fold higher concentration of intracellular porphyrin than shifted-up iron-sufficient cells at 24 hours after the addition of iron.

Extracellular porphyrin followed the trend set by intracellular porphyrin, as opposed to that of the cytochromes. No significant change in extracellular porphyrin concentration was observed on increasing the iron concentration above 25.0μgFe/ml, with both iron-deficient and iron-sufficient cells (Table 24). As with

TABLE 21

Cytochrome Concentration Of Iron-Deficiently Grown *M. smegmatis* In Further Shift-Up Experiments Using Higher Extracellular Iron Concentrations

Extracellular Iron Concentration (µg/ml)	Cytochrome Concentrations (nmol/g dry wt)					
	1h			24h		
	c	b	a	c	b	a
0.05	1.5	n.d.	n.d.	2.1	n.d	n.d.
2.0	2.5	2.5	n.d.	4.3	7.0	5.4
25.0	3.1	8.0	7.3	9.5	12.4	11.3
50.0	2.8	7.5	6.8	6.1	11.9	7.1
75.0	3.0	9.1	7.1	6.4	14.8	5.1
100.0	2.5	6.4	7.4	5.8	13.5	6.9
150.0	2.6	7.1	6.2	6.3	13.2	6.2

Legend:- Iron was added to 60h iron-deficient cells to give final iron concentrations from 2.0 to 150µgFe/ml. Samples were taken at 1h and 24h after the addition of iron for the determination of cytochrome concentration.

TABLE 22

Cytochrome Concentration Of Iron-Sufficiently Grown *M. smegmatis* In Further Shift-Up Experiments Using Higher Extracellular Iron Concentrations

Extracellular Iron Concentration (µg/ml)	Cytochrome Concentrations (nmol/g dry wt)					
	1h			24h		
	c	b	a	c	b	a
2.0	8.9	4.7	3.2	9.8	6.8	4.7
25.0	18.6	32.2	6.8	18.2	31.3	8.2
50.0	18.2	28.4	5.8	18.6	29.1	8.7
75.0	12.9	27.9	6.2	20.5	31.5	9.4
100.0	14,6	27.0	5.8	19.1	30.2	8.1
150.0	18.8	27.8	9.4	15.7	32.7	9.4

Legend:- Iron was added to 60h iron-sufficient cells to give final iron concentrations from 25.0 to 150µgFe/ml. Samples were taken at 1h and 24h after the addition of iron for the determination of cytochrome concentration.

TABLE 23

Intracellular Porphyrin Concentration Of *M. smegmatis* In Further Shift-Up Experiments Using Higher Extracellular Iron Concentrations

Extracellular Iron Concentration (µg/ml)	Intracellular Porphyrin Concentration (µmol/g dry wt)			
	Iron-Deficient Cells		Iron-Sufficient Cells	
	1h	24h	1h	24h
0.05	60	75	-	-
2.0	244	621	128	230
25.0	234	497	113	192
50.0	191	448	120	136
75.0	289	402	122	167
100.0	149	265	103	218
150.0	125	239	82	179

Legend:- Iron (as soluble iron) was added to iron-deficient and iron-sufficient cells to give final iron concentrations from 2.0 to 150.0µg/ml. Samples were taken at 1h and 24h after the addition of iron, for the determination of intracellular porphyrin concentration (as in Materials and Methods).

TABLE 24

Extracellular Porphyrin Concentration of *M. smegmatis* In Further Shift-Up Experiments Using Higher Extracellular Iron Concentrations.

Extracellular Iron Concentration (µg/ml)	Extracellular Porphyrin Concentration (µg/l) Iron-Deficient Cells 1h	24h	Iron-Sufficient Cells 1h	24h
0.05	1.2	2.6	-	-
2.0	5.4	61.1	19.7	55.3
25.0	2.9	268.9	5.5	27.0
50.0	3.5	316.6	5.6	44.5
75.0	2.4	288.7	5.4	28.5
100.0	2.9	291.7	6.9	33.2
150.0	0.5	263.8	2.2	20.7

Legend:- Iron was added to iron-deficient and iron-sufficient cells to give final concentrations from 2.0 to 150.0µg/ml. Samples were taken at 1h and 24h after the addition of iron, for the determination of extracellular porphyrin concentration (as in Materials and Methods)

the values obtained for intracellular porphyrin concentration, the highest extracellular porphyrin concentrations were obtained with shifted-up iron-deficient cells, which were up to 10-fold greater than the concentrations detected in shifted-up iron-sufficient cells.

Chapter 3 A Study Of The Value of Mycobactins And Exochelins As A Chemotaxonomic Character For Possible Differentiation of *M. avium* From *M. intracellulare*.

The use of mycobactin for taxonomic purposes, as already discussed (see Introduction) is a possible method of studying the overlap between the mycobacterial species *M. avium* and *M. intracellulare*. Eight strains of the *M. avium-intracellulare* complex were each grown iron-deficiently (as previously described) to stimulate maximum production of mycobactin. A wide variation in the cell yield and mycobactin concentration was observed between the different strains (Table 25). The properties of the mycobactins obtained from these strains were then compared using a wide variety of techniques e.g. t.l.c., n.m.r and h.p.l.c., to determine whether any similarities or differences between them followed a trend that represented a possible species boundary.

Thin Layer Chromatography (t.l.c.) Of Mycobactins

T.l.c. has already been used to distinguish between mycobactins from various species of *Mycobacteria* (see Introduction), and has the added advantage of requiring only crude mycobactin solutions. With the range of solvent systems used, the mycobactins from the *M. avium-intracellulare* complex were clearly distinguishable from mycobactins from other mycobacterial species, as illustrated by the large difference in mobilities of these mycobactins to the reference mycobactins employed (mycobactins S, P691, T, M and N). However, within the *M. avium-intracellulare* group itself, there were no significant differences in the

TABLE 25

Growth and Mycobactin Concentration
In Strains of the *M. avium-intracellulare* Complex

Species		Cell Dry Weight (g/100ml)	Mycobactin Concentration (mg/g dry wt)
M. avium NCTC 8559	(M1)	0.42	24.5
M. avium NCTC 8562	(M2)	0.42	6.3
M. avium Deer isolate	(M4)	0.53	1.5
M. avium Pig isolate	(M8)	0.32	7.4
M. intracellulare Ugandan Soil Isolate	(M12)	0.13	42.6
M. intracellulare Ugandan Soil Isolate	(M13)	0.19	1.1
M. intracellulare ATCC 13950	(M18)	0.22	22.3
M. intracellulare	(M34)	0.39	8.7

Legend:- The *M. avium-intracellulare* strains were grown iron-deficiently, harvested and cell dry weights and mycobactin concentration determined as previously described.

mobilities of the mycobactins that could be associated with possible species boundaries within the complex (Table 26).

This indicated that the mycobactins from this mycobacterial complex were similar as a group, as small differences in the structure of mycobactin usually produce measurable differences in their chromatographic mobilities e.g. mycobactins S and T, which differ only in the type of aliphatic chain in the R_1 substituent position, have different mobilities in some solvent systems. Thus as no differences between the mycobactins of the *M. avium-intracellulare* complex could be detected by t.l.c., the more refined technique of h.p.l.c. was applied to this problem.

High Pressure Liquid Chromatography (h.p.l.c.) of Mycobactins

The technique of h.p.l.c. has only recently been applied to the mycobactins; it has been established that mycobactins, purified by the usual techniques to 100% purity, can still be separated into at least six or more recognisably different components by this technique (Ratledge and Ewing, 1978). The basis for this separation is the presence of aliphatic chains of varying carbon length occupying the R_1 (or possibly R_4) substituent position on the mycobactin molecule. This mixture of carbon chain lengths within one type of mycobactin does not afford separation by t.l.c., and a high resolution technique such as h.p.l.c. is required to exploit these differences. It was hoped that the application of this technique to the mycobactins of the *M. avium-intracellulare*

TABLE 26

Thin Layer Chromatography of Mycobactins
From *M. avium-intracellulare* Species

Legend:-

Solvent System[a]

I Cyclohexane/tert-butanol (9:1, v/v)

II Isopropanol

III Petroleum spirit/n- butanol/ethyl acetate (2:3:3 by vol)

IV Benzene/ethyl acetate (1:1, v/v); triple development

V Benzene/ethyl/acetate (2:3, v/v).

VI Benzene/ethyl acetate (1:3, v/v); double development

VII Benzene/ethyl acetate (1:5, v/v)

VIII Benzene/ethyl acetate (1:9, v/v)

IX Benzene/isopropanol (1:1, v/v)

a. All t.l.c. was performed on silica gel (250μm thickness) apart from System I, which was performed on aluminium oxide (250μm thickness).

b. For solvent Systems IV and VI, theree and two successive developments respectively, were used.

TABLE 26 (Continued

Chromatographic Mobilities[a]

Source of Mycobactin	I	II	III	IV	V	VI	VII	VIII	IX
M. avium M1	0.73	0.54	0.90	1.00	0.30	1.0	0.40	0.40	0.76
M. avium M2	0.72	0.54	0.90	1.00	0.29	1.0	0.40	0.41	0.77
M. avium M4	0.71	0.54	0.92	1.22	0.33	1.06	0.44	0.46	0.78
M. avium M8	0.75	0.55	0.91	1.04	0.30	0.98	0.40	0.42	0.80
M. intracellulare M12	0.73	0.55	0.92	1.05	0.28	0.92	0.40	0.42	0.80
M. intracellulare M18	0.74	0.55	0.92	1.04	0.27	0.92	0.39	0.42	0.81
M. smegmatis 8548	0.16	0.31	0.36	0.12	N.D.	0.22	N.D.	N.D.	N.D.
M. bovis BCG(Glaxo P691)	0.26	0.43	0.57	0.18	N.D.	0.21	N.D.	N.D.	N.D.
M. tubuclosis H37Rv[b]	0.27	0.33	0.40	0.16	N.D.	0.22	N.D.	N.D.	N.D.
M. marinum b,c	0.53 0.64	0.36 0.58	0.43 0.65	0.41 0.84	N.D.	0.49 0.82	N.D.	N.D.	N.D.

a. For all systems except IV and VI, R_F values are given. For Systems IV and VI, R_R values (relative to the mobility of mycobactin M1) are given.

b. Mycobactins kindly supplied by G. A. Snow.

c. Two spots are visible, the slower running is the major component (mycobactin M); the other is mycobactin N.

N.D. = Not Determined.

complex would point out any species difference within the group.

The purified mycobactins examined by this technique each separated into four or five components, the relative proportions of which are shown in Table 27, along with the elution times for each components.

As can be seen from Table 27, there appeared to be no pattern or trend discernible that could be associated with the possible species boundary within the complex. Thus mycobactins from the *M. avium-intracellulare* complex have a non-specific distribution of their individual components, separable by h.p.l.c. This distribution, on the basis of the carbon lengths of the aliphatic chain at the R_1 (or R_4) substitutent position, may reflect differences in the availability of various alkyl chains to be inserted into the R_1 position. This may in turn, reflect the different environments of these organisms *in vivo*, and the supply of nutrients that is available to them (see Discussion).

As h.p.l.c. did not distinguish between the various mycobactins, a technique was required that would give more information about the alkyl chains on the mycobactin molecules. Gas liquid chromatography of the fatty acids isolated from the mycobactin molecules was therefore used, to determine whether any specific variation occurred in the substituents at the R_1 position.

Gas Liquid Chromatography (g.l.c.) Of Fatty Acids Obtained from *M. avium-intracellulare* Mycobactins

The fatty acids obtained from hydrolysis of the *M. avium-intracellulare* mycobactins were identified by g.l.c.

TABLE 27

Proportions And Elution Time For Components Separated from Mycobactins By h.p.l.c.

Elution Time (min)	Relative % Mycobactins*						
	AV1	AV2	Av4	AV8	Av12	Av18	AV34
0.5							21.5
0.8	61.7					4.0	
1.1			6.7			7.2	
1.3			7.3				67.9
1.9		35.4					
2.3	4.5			8.5	24.9	7.2	
2.6	10.7		73.8		26.0		7.9
3.0				3.4			
3.7						51.0	
4.0		24.2					
4.6				15.4	43.4		
5.3			6.1				
7.4	18.9		6.1				
8.5				66.6			
9.2		38.1					
10.1						30.7	
10.8							2.7
12.4					5.8		
19.3				6.2			
22.5	4.2	2.3					

Legend: Mycobactins were examined by h.p.l.c. as already described (see Materials and Methods). The figures show the proportions and elution times of each of the components.

* Mycobactins AV1-AV8 were from *M. avium* strains, while mycobactins AV12 - AV34 were from *M. intracellulare* strains.

TABLE 28

Distribution (by g.l.c. analysis) Of Methyl Esters Of Fatty Acids Isolated from *M. avium-intracellulare* Mycobactins

Relative Proportions (%, w/w) Of The Fatty Acids

Carbon Number	Possible Identity[b]	Mycobactins[a] Av1	Av2	Av4	Av8	Av12	Av13	Av18	Av34
8.0	$C_{8:0}$			26	20			39	
9.0	$C_{9:0}$						30		
10.0	$C_{10:0}$		5						
11.0	$C_{11:0}$			3	21	10			
11.3	Δ^2-cis-$C_{11:1}$	19		6					
12.0	$C_{12:0}$	7	17			19	4		
12.6	Δ^2-cis-$C_{12:1}$								9
14.0	$C_{14:0}$	29	13	14	39	17	6	10	13
14.6	$C_{14:1}$			12	4	11	27	8	
16.0	$C_{16:0}$	6	16	27	4	27	5	27	32
16.3	Δ^2-cis-$C_{16:1}$						5		
17.0	$C_{17:0}$	11				5			
17.6	$C_{17.1}$			4			5	11	19
18.0	$C_{18.0}$	16	33	8	12		6	4	12
18.3	Δ^2-cis-$C_{18:1}$		11				8		
19.6	$C_{19:1}$	12				11	4		15
20.0	$C_{20:0}$		5						

Legend:- Gas liquid chromatography of the methyl esters was carried out as previously described (see Materials and Methods).

[a]Mycobactins Av1-Av8 were from *M. avium* strains while mycobactins Av12-Av34 were from *M. intracellulare* strains.

[b]Under 'Possible Identity', identifications were made using appropriate standards of saturated and Δ^9- unsaturated fatty acids; Δ^2-cis-monounsaturated acids were not identified as such, but were presumed to be so by their retention times and the previously published data of White and Snow (1969). Many trace components were detected, but for reliable quantative analysis, components constituting less than 3% were ignored.

as already described (see Materials and Methods). The identity and relative proportions of the fatty acids are summarised in Table 28. Two main classes of fatty acid were observed: saturated and the unsaturated with one double bond. However most mycobactins examined were predominantly comprised of the saturated acids.

However, as was found from the data obtained using h.p.l.c., there appeared to be no relationship between the fatty acids from the *M. avium* and *M. intracellulare* mycobactins that would enable this complex to be separated into two species. Thus as this technique did not distinguish between the various mycobactins, a technique was required that would yield more information about the structure of the mycobactins. N.m.r. spectroscopy was therefore used as this can identify the various R groups on the mycobactin nucleus that serve to characterise a particular mycobactin.

Nuclear Magnetic Resonance Spectroscopy (n.m.r.) of Mycobactins

N.m.r. spectroscopy was performed on the metal-free mycobactins, as previously described (see Materials and Methods). As this technique has been used in a study of a large number of mycobactins (Greatbanks and Bedford, 1969), it was possible to identify the substituent R groups on the mycobactin nucleus by using the data provided in this work. As controls, the n.m.r. spectra were determined for two mycobactins that had been incorporated into the study of Greatbanks and Bedford, mycobactins P and S.

The n.m.r. spectra obtained from the *M. avium-intracellulare* mycobactins, as well as mycobactins P and S, are included (Appendix I) and the deduced identity of the main peaks summarised in Table 29. On examination of the spectra, no significant differences between them were observed apart from an unexplainable resonance at 4.0 p.p.m. in the mycobactins obtained from the *M. intracellulare* strains (see below). The R_1 substituent group was an alkyl chain, demonstrated by a large peak at 1.2 - 1.3 p.p.m., seen in all the spectra. This is a characteristic of most mycobactins, although the alkyl chain can occur at the R_4 substituent position (mycobactins M and N, and the nocobactins) but this is comparatively rare. The R_2 substituent was identified as a hydrogen in all cases, due to the peak at 7.6 p.p.m. present in all the spectra. The R_3 substituent was shown to be a methyl group by the presence of a doublet at 1.5 - 1.6 p.p.m. in all the spectra. The R_5 substituent was inferred to be a methyl group, which would have a peak at 1.15 p.p.m. (a doublet), but this would be obscured by the much stronger signal from the alkyl chain. If however, a hydrogen was to occupy this position, an absorption peak at 2.5 p.p.m. would be seen. As no such peak could be seen in the n.m.r. spectra of any of the *M. avium-intracellulare* mycobactins, this was confirmatory for the presence of a methyl group at the R_5 position. No substituents other than a methyl or a hydrogen have every been detected in this position on the mycobactin nucleus.

TABLE 29

Identification Of Peaks In The n.m.r. Spectra Of Mycobactins

Resonance (p.p.m.)	Identity
0.85 - 0.90	Protons on terminyl methyl (all mycobactins)
1.2 - 1.3	Protons in aliphatic chain (R_1) (all mycobactins)
1.5-1.6	Methyl group (R_3) in *M. avium-Intracellulare* mycobactins.
2.5	Methyl group (R_2) in mycobactin P and a proton (R_5) in mycobactin S; not seen in *M. avium-intracellulare* mycobactins.
4.0	Unexplainable absorption peak; only seen in *M. intracellulare* mycobactins (see text)
5.9	CH = CH (all mycobactins)
7.3	Protons in benzene ring (all mycobactins)
7.6(d)	Proton (R_2) in mycobactin S and in *M. avium-intracellulare* mycobactins.

Legend:- N.m.r. spectra of mycobactins determined as indicated (see Materials and Methods).

Other peaks, not mentioned above, represent the protons on various parts of the molecule that are common to all mycobactins.

(d) = doublet.

The identity of the R_4 substituent was not certain from this evidence. It could possibly be a hydrogen or a methyl group, although the latter was unlikely. However the n.m.r. spectra of the mycobactins from the *M. intracellulare* strains all possessed a peak at 4.0 p.p.m. which could not be explained using the n.m.r. data provided by Greatbanks and Bedford (1969). This peak was absent from all the *M. avium* strains examined. This singular difference between the n.m.r. spectra of the mycobactins from the *M. avium* and *M. intracellulare* strains may result from the moiety occupying the R_4 substituent position being different for the two groups of organisms, although this cannot be established from this n.m.r. data.

For this reason an attempt was made to hydrolyse the mycobactin molecule and isolate the fragment that contained the R_4 group. This fragment would be examined by n.m.r. and mass spectrometry, whereby the identity of the R_4 group could be established. Simultaneously, mass spectrometry of the whole mycobactins was attempted to determined the molecular weight and to provide structural evidence to corroborate with the data already gained.

Attempted Degradation Of Mycobactins and Isolation of Cobactin

Degradation of the mycobactin molecule was attempted as described (see Materials and Methods) in order to isolate the fragment of the parent mycobactin (termed cobactin) that contained the R_4 substituent. However

the cobactins obtained from the degradation of mycobactins Av2 and Av34 gave poor spectra when examined by n.m.r. (Figures 21 and 22). These spectra contained no structural data and gave no information as to the identity of the R_4 substituent (D. F. Ewing, personal communication). Purification of the cobactins was attempted as described (see Materials and Methods), but the spectra obtained using the purified material did not contain any structural information.

The failure of this attempt to elucidate the identity of the R_4 substituent may lie with the amount of mycobactin available for degradation. The cleavage of mycobactin in the correct position to obtain the intact cobactin fragment is difficult to achieve without causing partial cleavage at other parts of the molecule. Consequently the original method (Snow, 1954) started with over 5g of mycobactin, which facilitated the production of enough cobactin for examination. However production of this amount of mycobactin was impracticable in this study and only 500mg of mycobactin were available for degradation.

Mass Spectrometry Of Mycobactins

Before committing the mycobactins from the *M. avium-intracellulare* strains, mycobactins P and S were examined by mass spectrometry. These mycobactins had been studied by previous workers using this technique (Snow and White, 1969: White and Snow, 1969). Unfortunately, although using the same make of equipment and operating conditions as the above workers, no parent

ion could be detected. Mass spectrometry of mycobactins P and S was attempted using both the metal-free and aluminium forms, without success.

Using the conditions described (see Materials and Methods) molecular ions over 700 could not be detected, and the peaks from ions at 700 were 10^3- to 10^4- fold weaker than lower weight molecular ions (A. Roberts, personal communication). Varying both the ionisation temperature and the ionisation voltage did not enable the parent ion to be detected. As mass spectrometry of these mycobactins has been carried out by the above workers, no explanation can be offered as to the failure to repeat this technique.

Exochelin Production By *M. avium-intracellulare* Strains

The spent growth medium from each strain used was examined for the presence of exochelin, which is normally produced extracellularly by *Mycobacteria* during iron-deficient growth. In all cases an exochelin was detected which could be extracted (as the ferric complex) into chloroform. To ascertain that the material isolated from the spent growth medium was not mycobactin, the exochelins and mycobactins isolated from the *M. avium-intracellulare* strains were examined together by t.l.c. Using benzene/ethy/acetate (1:5,v/v) as the solvent system, all the *M. avium-intracellulare* mycobactins move with the solvent front, while the exochelins remain at the origin. By examination of the t.l.c. plates under u.v. light, the mycobactins were seen as u.v. absorbent spots whereas the exochelins were not. No fraction of mycobactin or exochelin on t.l.c. corresponded to the opposing mycobactin or exochelin obtained from the same strain.

Chapter 4 Growth of *Mycobacterium paratuberculosis* And Mycobactin-Dependent *Mycobacterium avium*

As already discussed, the fastidious growth requirements of this group of Mycobacteria have only been met by the addition of mycobactin to the growth medium of these organisms (see Introduction). The purpose of the experiments carried out in this chapter were to determine whether these Mycobacterial species produce even small amounts of mycobactin and exochelin during growth. However detection of a mycobactin if produced, would be complicated, as it could possibly be difficult to separate this mycobactin from that used to promote growth. Consequently it was first necessary to determine whether any compounds other than mycobactin could be used to promote the growth of these organisms.

Growth of Mycobactin-Dependent Species

Three mycobactin-dependent *M. avium* strains (M21, M22 and M24) and one *M. paratuberculosis* strain (M27) were grown as indicated previously (see Materials and Methods). The materials used to support growth are shown in Table 30. Mycobactins S and Av (the mycobactins from *M. smegmatis* and *M. avium*, respectively) were included as controls.

The other materials included were chosen because of their role in iron transport, or their reported function as growth-promoting agents for these mycobactin-dependent species (see Introduction).

Nocobactin 3318 supported the growth of both *M. paratuberculosis* and mycobactin-dependent *M. avium* to the same extent as, if not better than, the two mycobactins included in the study (Table 30). No other compound tested was able to support the growth of

TABLE 30

Growth Of Mycobactin-Dependent *M. avium* and *M. paratuberculosis* On Various Growth-Promoting Agents

Addition to Growth Medium	Growth M21	M22	M24	M27
Mycobactin S	4	3	5	0.147
Mycobactin Av	4	3	5	0.169
Exochelin MS	0	0	3	0.008
Nocobactin 3318	4	3	5	0.193
Ferric Ammonium Citate	2	2	2	0.004
Salicylic Acid	3	2	4	0.010
Vitamin K	0	0	0	0.007
No Addition	0	0	0	0.008

Legend:- All growth-promoting agents were incorporated into the Dubos Broth growth medium at a final concentration of 1µg/ml.

M21, M22 and M24 refer to the three mycobactin-dependent *M. avium* strains examined.

Growth was estimated for these strains on a visual basis, where 0 denotes no growth, while 5 denotes pellicle formation that covered the surface of the growth medium.

M27 refers to the *M. paratuberculosis* strain employed. Growth was estimated as cell dry weight (g/100ml) as in Materials and Methods.

M. paratuberculosis, while salicylic acid was able to support the growth of the mycobactin-dependent *M. avium* strains to a limited extent. Notable was the failure of exochelin MS to support the growth of these organisms (see Discussion). Nocobactin 3318 was therefore chosen as the material to be used for the future growth of these mycobactin-dependent species. Nocobactin could be easily seperated from mycobactin by t.l.c. and therefore presented no problem to the detection of a mycobactin, if produced.

Growth of Mycobactin-Dependent Species on Nocobactin 3318

One mycobactin-dependent *M. avium* strain (M24) and one *M. paratuberculosis* strain (M27) were grown as before on ferrinocobactin 3318 at 1µg/ml, for the detection of mycobactin and exochelin. The cells and the spent growth medium were examined as indicated previously (see Materials and Methods).

(i) Examination of Cells

Both *M. avium* and *M. paratuberculosis* cells, when extracted into ethanol, were found not to contain any detectable mycobactin (the limit of detection for mycobactin is approximately 25µg/ml). Nocobactin 3318 was also absent from the ethanol extracts of both organisms. When further concentrated and applied in total to a t.l.c. plate (solvent system of butanol/ethyl acetate/pet. spirit, 2:3:3, by vol), no material corresponding to either mycobactin or nocobactin was observed. Further, on spraying the t.l.c. plate with bipyridyl reagent, no red material was seen (this reagent enabled amounts of mycobactin or nocobactin

as small as 100µg to be detected).

(ii) Examination of The Spent Growth Medium

The chloroform extract of the spent growth medium of both organisms, (to extract any nocobactin 3318, and any chloroform-soluble exochelin produced) was examined spectrophotometrically, but neither an exochelin nor the original nocobactin could be detected. As above, on further concentration and application in total to a t.l.c. plate, neither an exochelin nor the nocobactin were observed. Neither material was detected on spraying the t.l.c. plate with bipyridyl reagent.

On analysis of the ether extracts of the spent growth medium by spectrophotometry, that from mycobactin-dependent *M. avium* contained salicylic acid. Salicylic acid was not detected in the ether extract of the spent growth medium of *M. paratuberculosis*. This was confirmed by the mobility of salicylic acid on ascending paper chromatography (see Materials and Methods). Both extracts were run against salicylic acid (R_F = 0.70) which exhibits a light blue flourescence under u.v. light. By this method, salicylic acid was observed in the ether extract of the spent growth medium of mycobactin-dependent *M. avium* but not in that of *M. paratuberculosis*.

DISCUSSION

Chapter 1 Studies On Ferrimycobactin Reductase

(i) Activity and Non-Specific Nature of Ferrimycobactin Reducatase

For the reasons already outlined it would not be surprising to find reduction as a method of intracellular release of iron, common in microbial systems. At this time however, only one organism other than *M. smegmatis*, that is *Neurosporra crassa* (Ernst and Winkelmann, 1977b), has been shown to possess an enzyme responsible for reduction of the iron-chelate which is involved in iron transport. Reduction was implicated as the method of release of iron from ferrimycobactin in *M. smegmatis*, as the intact chelating agent could be recovered after the enzyme assay had been carried out (Table 2). Intracellular release of iron by reduction is a favourable mechanism for micro-organisms as the iron is in a form ready for insertion into suitable acceptor molecules e.g. non haem iron proteins, haem precursors, while the chelating agent is left intact and can continue to function. However this method can also release iron prior to demand, where it can be subject to hydroxylation and precipitation. In such circumstances, one would assume the activity of the enzyme involved to be such that release of iron would be concomitant with demand. This would account for the low activity of ferrimycobactin reductase observed in the cell free extracts, namely 0.2 - 0.4 nmol reduced $min^{-1}mg\ protein^{-1}$, seen by myself and earlier workers (Brown and Ratledge, 1975).

That the affinity of this enzyme for ferrimycobactin was unmeasurably high is not surprising considering the likely concentration of ferrimycobactin *in vivo*. Although the mycobactin concentration is high (in iron-deficient cells), the concentration of the ferri-form will be low. Thus the high affinity of this enzyme for ferrimycobactin would enable the organism to reduce the iron at the maximum rate of the enzyme even at low ferrimycobactin concentrations. However if mycobactin were employed by *M. smegmatis* as a 'store' for iron (see 'Role of Ferrimycobactin Reductase in Iron Transport'), then high ferrimycobactin concentrations could be attained on some occasions. Values of 10mM could be achieved, assuming the intracellular volume of *M. smegmatis* is 2ml (g dry wt)$^{-1}$ (a typical value for bacteria e.g. Midgley and Dawes, 1973) if all the mycobactin present in iron-deficient cells was converted to the ferri-form. Presumably under these conditions, the low level of enzyme activity would prevent intracellular release of iron prior to demand. This is illustrated by the rate of release of iron from ferrimycobactin in *M. smegmatis*, as shown in Figure 6. In this case, a large proportion (approximately 45%) of the mycobactin in the cell was in the ferri-form, yet the rate of release of iron into the cell was of the same order of magnitude as observed *in vitro*. This low rate of release of iron with high ferrimycobactin concentration is in keeping with the idea of mycobactin as a store for iron, where iron is released at a rate equal to demand. This would prevent hydroxylation and precipitation of any iron excess to requirements; this is discussed more fully

in the 'Role of Ferrimycobactin Reductase in Iron Transport').

It is possible that, although the ferrimycobactin concentration is high, the concentration available to the enzyme *in vivo* is low. Reduction of ferrimycobactin presumably occurs at the interface of the cell envelope and cytoplasm, where once the concentration of ferrimycobactin is sufficient to saturate this interface, then further increases in the ferrimycobactin concentration will not increase the concentration available to the enzyme. This may account for the 4 to 5- fold lower value for the rate of release of iron from ferrimycobactin observed *in situ* compared to that seen *in vitro*.

The finding of ferrimycobactin reductase activity however, in extracts of *Candida utilis* and *Escherichia coli* at the same level of activity observed in *M. smegmatis*, questions the specificity of this enzyme (Table 6). Similarity between the activities obtained from all these organisms is highlighted by the action of the sulphydryl inhibitor, iodoacetamide, on the activity of ferrimycobactin reductase in cell free extracts prepared from each organism. Ferrimycobactin activity could also be catalysed *in vitro* by the use of reagents containing reduced thiol groups (Table 7), as well as yeast alcohol dehydrogenase, whose activity in reducing ferrimycobactin was surmasied to be dependant upon the reduced thiol groups on the molecule.

In the light of these findings, the activity observed as ferrimycobactin reductase is probably not due to an enzyme specifically synthesised by *M. smegmatis* for this purpose. Activity is thus possibly catalysed by one or more enzymes, whose broad specificity will allow the

reduction of iron bound to mycobactin. This certainly accounts for the observation that activity could be found in cell free extracts of iron-sufficient *M. smegmatis* (as iron-sufficient cells have an undetectable amount of mycobactin) as well as *E. coli* and *C. utilis*. Additionally, as ferrimycobactin is not the natural substrate for these enzymes, the broad specificity in terms of iron-containing compounds reduced is also accounted for (Table 8).
The finding of an 'iron reductase' in membrane preparations from *E. coli, R. spheroides, S. itersonii* and other organisms (Dailey and Lascelles, 1977) and *S. aureus* (Lascelles and Burke, 1978) may be related to that seen above in *M. smegmatis, E. coli* and *C. utilis*. The 'iron reductase' observed in the above organisms was similar to the ferrimycobactin reductase of *M. smegmatis*, in that it was inhibited by oxygen and could use NADH as an electron donor. Activity was shown to be due to the reduction of iron by reduced components of the respiratory chain, but in the presence of oxygen, these components were preferentially oxidised by successive members of the respiratory chain, rather than by the iron-containing compounds (Dailey and Lascelles, 1977; Lascelles and Burke, 1978). Reduction of iron by components before cytochrome c has been suggested by these workers on the basis of inhibition studies; the interaction of the ferric ion with the respiratory chain may possibly occur by exchange with forms of non-haem iron e.g. iron-sulphur proteins.

However the 'iron reductase' is unlike the ferrimycobactin reductase mentioned above and coprogen reductase (Ernst and Winkelmann, 1977a,b) in that it cannot

reduce other naturally occurring iron-containing compounds e.g. ferri-ferrioxamine B. While not synonymous with 'iron reductase', ferrimycobactin reductase activity may occur by a related mechanism. The strong electronegativity of ferrimycobactin (Table 3) would indicate that reduction would take place prior to cytochrome b, if respiratory chain component(s) were involved. Further, the involvement of non-haem iron proteins in this process(similar to that postulated by Lascelles and Burke, 1978, for the mode of action of 'iron reductase') is also possible.

Non-haem iron has been shown to be involved in the respiratory pathways of *M . phlei* (Kurup and Brodie, 1967) and *E. coli* (Kim and Bragg, 1971) so its involvement in the respiratory chain of *M. smegmatis* is to be expected. Additionally, the redox potential of some non-haem iron proteins e.g. iron-sulphur proteins is known to be more negative than that of ferrimycobactin (up to -500mV; Neilands, 1974).

The effects of some inhibitors on the reduction of ferrimycobactin (Table 4) would support such a hypothesis. The inhibition by potassium cyanide of ferrimycobactin reduction would indicate the inhibition of electron transfer from NADH to ferrimycobactin via component(s) of the respiratory chain. Further the inhibition of ferrimycobactin reductase by sulphydryl reagents implicates the involvement of thiol groups in this process, as was observed with the reduction of ferrimycobactin using thiol-containing reagents (Table 7). This may also imply the involement of iron-sulphur proteins in this process.

The activity of ferrimycobactin reductase is thus probably associated with protein(s) whose main function is not that of a siderophore reductase. This protein may be involved with the respiratory chain, as discussed above, in a manner similar to that observed with 'iron reductase' (Lascelles and Burke, 1978). However the role of ferrimycobactin reductase activity in iron trasport in *M. smegamtis* is uncertain especially in view of the recent findings that mycobactin is not involved in iron uptake when iron at physiological concentrations is presented to the cell as ferriexochelin (Stephenson and Rateledge, 1979). In view of such findings, the role of both mycobactin and ferrimycobactin reductase in iron transport are discussed in the following section,

(ii) Role of Mycobactin And Ferrimycobactin Reductase In Iron Transport

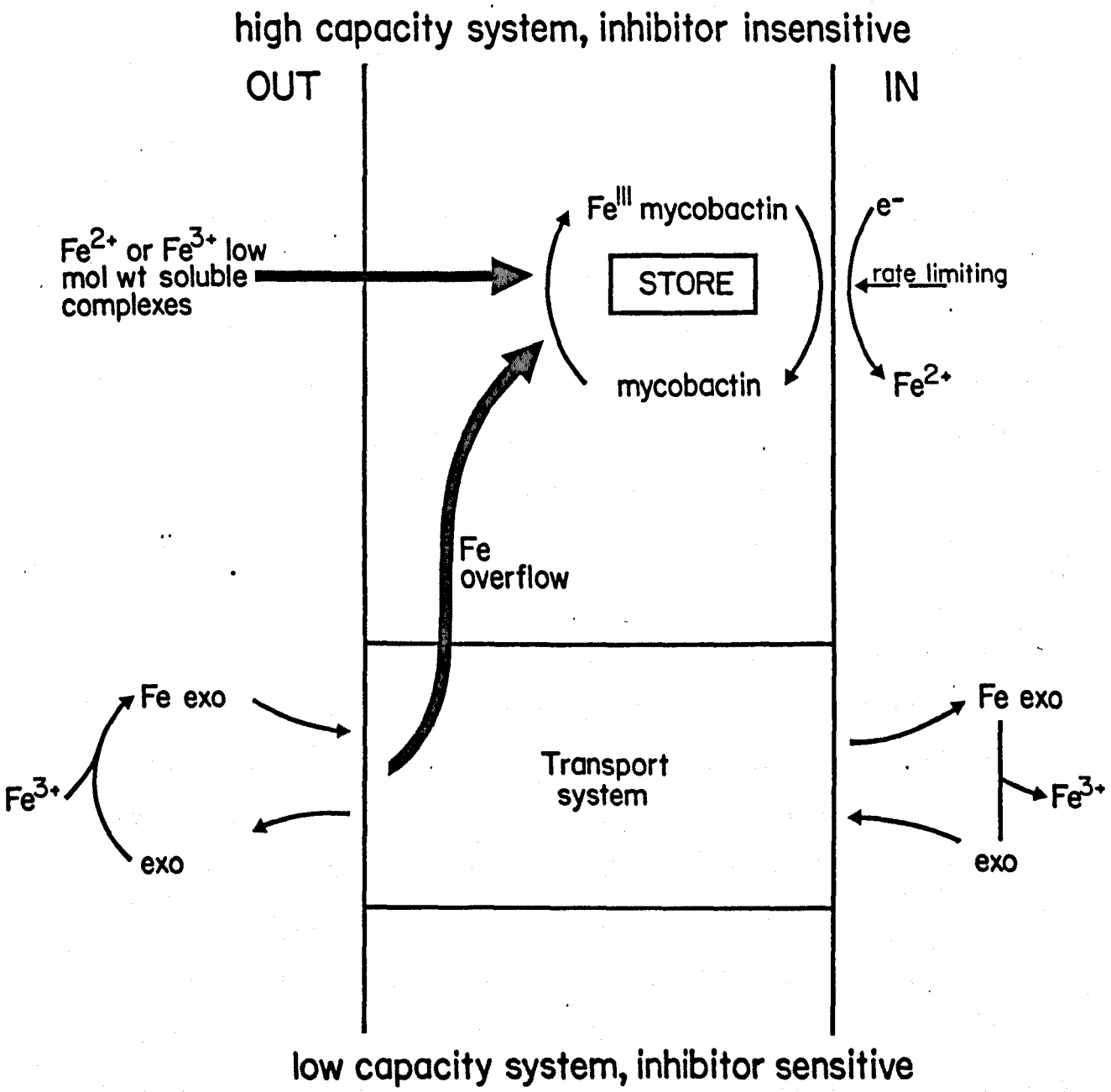
Due to the apparent non-specific nature of ferrimycobactin reductase, the role of this activity in iron transport is uncertain. Certainly, iron-uptake by *M. smegmatis*, when presented with iron at physiological concentrations, in the form of ferriexochelin, proceeds independantly of mycobactin. Transport by this mechanism is inhibitor sensitive and displays the characteristics of an active transport system (Stephenson and Ratledge, 1979). However when high ferriexochelin concentrations are presented to the cell, the process becomes inhibitor insensitive and resembles that which occurs when iron is presented to the cell as ferrisalicylate (where iron is known to be taken up via mycobactin; Stephenson and Ratledge, 1979). From

this, it appears that at high ferriexochelin concentrations, iron can be taken up via mycobactin, as well as via the exochelin mediated transport system. From this, it is possible to consider mycobactin as a 'store' or 'overflow' for iron, where iron uptake, when exceeding the capacity of the exochelin mediated system, will proceed via mycobactin (as seen above).

A mechanism to account for such a hypothesis, where mycobactin can be considered as an 'overflow', is shown in Scheme 2 (C. Ratledge, unpublished data). In this case, iron transport will occur via exochelin under normal growth conditions i.e. when the exochelin concentration is high but the available iron, and hence the ferriexochelin, concentration is low. It is only during conditions of excess available iron, or times of 'feast', to use the definition of Koch (see Introduction), that iron uptake will proceed via mycobactin.

Such a mechanism is also supported by the data obtained during the 'shift-up' experiments (see Discussion, Chapter 2). Under these conditions, iron uptake would occur via mycobactin on comparatively rare occasions *in vivo*, thus the necessity for a specific enzyme for the reduction of ferrimycobactin in order to release the iron is removed. In addition, the low activity observed for the reduction of ferrimycobactin would ensure that iron would be released intracellularly at a slow rate, as iron would already be entering the cell via the exochelin mediated system. This would prevent any tendancy for precipitation of unchelated iron that would occur if the intracellular release of iron from ferrimycobactin was greater than demand.

Scheme 2 Proposed 'Overflow Mechanism' of Iron Transport in *M. smegmatis*.



The observation that transfer of iron can occur from ferriexochelin to desferrimycobactin at an aqueous organic interface in the absence of any other agent (M. C. Stephenson, unpublished data), may indicate that transfer of iron from ferriexochelin to mycobactin could occur *in vivo*. If ferriexochelin were to associate with the cell wall prior to uptake of iron, it is possible that on saturation of the exochelin mediated iron uptake system, the greater affinity of mycobactin for iron would be sufficient to promote transfer of iron from ferriexochelin to mycobactin. As for the reduction of ferrimycobactin, this transfer of iron would also rely on a non-specific reductive system, as occasions when iron-uptake would occur via this process would be comparatively rare *in vivo*.

The observation that ferriexochelin can be reduced using cell free extracts prepared from *M. smegmatis* is interesting, as this could present a 'unified' picture of the intracellular release of iron in this organism. Using reduction as a method of release of iron is advantageous for the reasons already outlined. However proof of this system operating *in vivo* has yet to be obtained, but one would expect the organism to synthesise a specific mechanism for the intracellular release of iron from ferriexochelin and not rely on a non-specific system. However the rate of iron uptake from ferriexochelin by intact cells (37-72μmolFe $min^{-1}.g$ dry wt^{-1}; Stephenson and Ratledge, 1979) can be accounted for by the activity of this reductive process when catalysing the reduction of ferriexochelin *in vitro* (Table 18). The probable situation

is that there is a specifically synthesized system for the intracellular release of iron from ferriexochelin, which is paralleled by this non-specific process. If reduction by either this non-specific, or a specific process were to occur, then exochelin would be reutilised by the cell, but at this time it has been impossible to prepare radioactive exochelin of a sufficiently high specific activity to test this hypothesis (Stephenson and Ratledge, 1979).

The ability for salicylate to act as an acceptor of ferrous ions in the ferrimycobactin assay procedure (Table 8).in place of EDTA may reflect the situation *in vivo*. It is possible that salicylate may act as a 'trap' or 'acceptor' of ferrous ions on the reduction of ferrimycobactin at the interface of cell envelope and cytoplasm, and function to 'shuttle' ferrous ions to suitable acceptor molecules e.g. haem precursors. If this were so, it would necessitate anaerobic conditions in this region of the cell, to prevent the re-oxidation of ferrous to ferric salicylate. In addition, it would explain the enigmatic presence of salicylate in high concentrations, both intra- and extracellularly in iron-deficient *M. smegmatis*. Shown not to be responsible for the extracellular acquisition of iron (Ratledge *et al.*,1974), the function of salicylate has been questioned for some time. That salicylate has a function to fulfill other than as a precursor of mycobactin, was established by the isolation of a mutant of *M. smegmatis* that required salicylate for growth, yet could not be grown by the substitution of mycobactin (Ratledge and Hall, 1972).

That the production of salicylic acid increases with the onset of iron-deficient growth (Ratledge and Winder, 1962; 1966) may also link its, as yet unknown function with that of intracellular release of iron.

The ability of mycobactin (and to a lesser extent salicylic acid), to promote the growth of mycobactin-dependent species, and the failure of exochelin to promote growth (Table 30) may reflect the role that mycobactin has to play in *Mycobacteria*. If the growth requirements of these species were caused by an inability to acquire or 'scavenge' iron, then exochelin would be expected to promote growth. That it cannot, would indicate that the cause of the 'deficiency' in these organisms is not merely acquisition of iron. Neither is growth enhanced by the addition of a soluble source of iron, otherwise additions to the growth medium of compounds such as ferric ammonium citrate would have some effect on growth. The growth promoting properties of mycobactin (and the similar compound, nocobactin) and the inability of the other compounds employed to promote growth, would suggest that mycobactin does have a role to play in the iron metabolism of *Mycobacteria*.

Chapter 2 Growth Of *M. smegmatis* Under Varying Iron Concentrations

(i) Concentrations of Metabolites

The growth of any micro-organism *in vivo* will be subject to variations in the supply of nutrients, especially iron, whose insolubility in natural systems has already been discussed. These fluctuations in essential nutrients such as iron will force the cell to adapt in order to survive with a minimum amount of iron. Under such conditions, non-essential iron-containing compounds will be decreased in concentration or not produced at all during times of reduced nutrient supply. This enables the organism under these circumstances to maximize its chance of survival during stringent growth conditions, by conserving the small amount of the essential nutrient available for the production of materials necessary for the existence of the cell. This may result in changes in metabolic pathways or in energy coupling mechanisms. Obviously if the supply of nutrient falls below the minimum level which the organism can adapt to, then growth will cease.

As can be seen (Table 9), iron-deficient *M. smegmatis* contained approximately 25 times less iron per cell than when grown iron-sufficiently. As a result of this, changes in the concentrations of various intracellular iron-containing compounds were inevitable, but it is important to see where the organism has adapted most in order to survive at this lower iron concentration. The concentrations of cytochromes during iron-deficient growth were at most 5- to 7- fold lower than during iron-sufficient

growth (Table 10), whereas the difference in iron concentration between the two growth states was 40- fold. This is presumably to be expected with organisms such as *M. smegmatis*, which are obligate aerobes, and thus require cytochromes for survival. In this case one would expect the concentration of cytochromes to be maintained during iron-deficient growth, and the concentrations observed are probably the lowest that this organism can survive with. Further reductions in the extracellular iron concentration would presumably prevent the growth of this organism, as it could not survive with the lower cytochrome concentrations resulting from adapting to the new iron concentration.

The differences observed in the concentrations of the various cytochromes between the two growth states were interesting, as flavoprotein and cytochrome c were affected to a much lesser extent than cytochromes b and a (Table 10). These disproportionate changes in cytochrome concentration have been seen in many organisms (see Light and Clegg, 1974), and may reflect either a lack of co-ordinated control over their synthesis, or possibly altered pathways of electron transport during iron-deficient growth. The latter may certainly be true when considering the important role played by non-haem iron in respiratory chains (see Bragg, 1974). The 25- fold difference in non-haem iron concentration between iron-deficient and iron-sufficient *M. smegmatis* (Table 9) suggests that the concentration of non-haem iron in the respiratory chain may be reduced during iron-deficient growth. Impairment of respiratory function would also

account for the slower growth of *M. smegmatis* under iron-deficient conditions (Table 9). However the increased production of cytochrome b during growth with excess iron (Tables 21 and 23) indicates that a lack of co-ordinated control may also occur. Certainly cytochrome b has been the most effected by iron-deficiency in a number of other organisms (see Introduction), so the observation that cytochromes b and a are more depleted during such growth is not out of keeping with this.

The maintained cytochrome concentrations during iron-deficient growth of *M. smegmatis* would indicate that other iron-containing compounds in the cell were severely depleted. This has been seen with haem iron containing compounds, such as the enzymes catalase and peroxidase, which are greatly depleted during growth of this organism and many others (see Introduction). This would indicate that within the pool of iron in the haem iron fraction, certain haem iron-containing compounds will be depleted e.g. catalase and peroxidase, to maintain the concentrations of others e.g. cytochromes. The fraction of the intracellular iron present in the non-haem iron pool was almost the same during iron-deficient and iron-sufficient growth, although it declined more quickly during iron-deficient growth (Table 9). This would imply that under both sets of conditions, a certain proportion of the assimilated iron will enter the haem iron fraction; this proportion does not increase with the onset of iron-deficiency, so consequently, the concentrations of some haem iron compounds are heavily depleted to maintain the levels of others.

Non haem iron compounds must also follow the same trend as observed with haem iron compounds; some non haem iron compounds are severely affected during iron-deficient growth e.g. succinate dehydrogenase, ferredoxin (see Introduction) which would allow for the concentration of certain non haem iron compounds e.g. essential components of the respiratory chain, to be maintained. Again this is in keeping with the distribution of iron between the haem and non-haem fractions remaining constant during iron-deficient and iron-sufficient growth. The cell, rather than maintaining the concentration of one 'class' of iron-containing compound, maintains the levels of certain essential compounds within each 'class' of iron-containing compounds (see Introduction).

The uptake of iron by iron-deficient *M. smegmatis* would also support this. On presenting iron in various physiological forms to iron-deficient cells, all the iron was taken up, but again a certain distribution ratio was maintained between the amount entering the non-haem iron and haem iron fractions (Table 12). As before, approximately 70% of the iron entered the non-haem iron fraction initially, the other being channelled into the haem iron fraction. This was relfected in the gradual increase in concentration of the cytochromes (Tables 14, 15 and 16), and presumably the faster increase of other haem iron compounds e.g. catalase and peroxidase.

The concentrations of porphyrin during iron-deficient and iron-sufficient growth (Table 11) indicate an unusual method of control over the haem biosynthetic pathway. As already discussed (see Introduction) most organisms produce

excess porphyrin during iron-deficient growth, when the control by haem over the pathway is relaxed. However the production of excess porphyrin during iron-sufficient growth of *M. smegmatis* appears to be novel amongst micro-organisms (see Granick and Beale, 1978). Control over this pathway is usually at the level of one of the first two enzymes in the pathway; the absence of any significant difference in the activity of δ-aminolevulinic acid dehydratase (the second enzyme of the pathway) would imply that the site of control was perhaps the first enzyme of the pathway, δ-aminolevulinic acid synthetase. However, the failure to detect this enzyme means that this hypothesis cannot be substantiated; the failure to detect activity was attributed to the extreme instability of this enzyme, which has been acknowledged by a large number of other workers (see Results, Chapter 2.). Although alternative mechanisms for the production of δ-aminolevulinic acid have been proposed, utilising glutamic acid, (rather than succinyl-coenzyme A and glycine), these new mechanisms are believed to be confined to plants, and not present in microbial systems (Beale 1976; Granick and Beale 1978).

Regulation of haem and porphyrin synthesis in *M. smegmatis* cannot be by the usual mechanism i.e. feed back inhibition by haem, but by some more subtle mechanism, as yet unknown in microbial systems. Iron itself, or an iron-containing compound may actually stimulate the production of porphyrin by activation of the enzyme δ-aminolevulinic acid synthetase; certainly this mechanism prevents the needless flow of carbon through this pathway during iron-deficiency, preventing the production of unwanted

compounds. It is also possible that the concentrations of glycine and succinyl-coenzyme A will become depleted during iron deficient growth due to the production of mycobactin. Acyl-coenzyme A compounds are believed to be involved in the attachment of the aliphatic chain to the mycobactin molecule,while serine (which can be derived from glycine) is used for the synthesis of the oxazoline ring in the mycobactin nucleus (Brown, 1975). However such a mechanism as the sole control over haem and porphyrin synthesis is unlikely, as one would expect a more refined mechanism controlling the production of essential compounds such as the cytochromes. Alternatively, δ-aminolevulinic synthetase may be an iron-containing enzyme and thus only functional under iron-sufficient conditions. During iron-deficient growth, the reduction in iron concentration would prevent the formation of the active enzyme to the same extent as during iron-sufficient growth, and thus account for the reduced porphyrin concentration. However no iron-containing δ-aminolevulinic acid synthetase has yet been discovered in microbial systems (Granick and Beale 1978).

The rapid production of porphyrin (both intracellular and extracellular) after the addition of iron to iron-deficient *M. smegmatis* (Figures 9,and 10) illustrates the control exerted by iron over this pathway. Cytochrome concentrations, as described above, also rise on the addition of iron to iron-deficient cultures (Tables, 14, 15 and 16), in keeping with this observation. Another unusual feature of control over this pathway is illustrated by the change in the cytochrome and porphyrin concentration in *M. smegmatis* when

presented with large, non-physiological quantities of iron. The increased production of porphyrin by iron-deficient cells compared to iron-sufficient cells, on the addition of large quantities of iron (Tables 23 and 24) and the reverse trend observed with cytochrome synthesis (Tables, 21 and 22) may show other differences in the control of this pathway during iron-sufficient and iron-deficient growth.

(ii) Response of *M. smegmatis* To Added Iron

The response of iron-deficient *M. smegmatis* to added iron appeared to be dependent upon the form in which the iron was presented to the cell. As stated, on the addition of iron as "soluble" iron ($FeSO_4$), the cells were observed to turn a cherry-red colour, which was not seen when iron was added in an insoluble form ($Fe(OH)_3$) or in the physiological form of ferriexochelin. Some effects of the added iron appeared to occur more rapidly with the addition of iron as"soluble"iron: both intracellular and extracellular porphyrin concentration appeared to increase more rapidly when iron was added as "soluble" iron (Figures 9, and 10), while the decline in mycobactin concentration also occurred more rapidly when iron was added in this manner (Figure 8). It is possible that because of the very rapid binding of the added "soluble" iron to the mycobactin (giving the cherry-red colour within several minutes), the effect of the added iron would be observed quicker than with iron added as "insoluble" iron or ferriexochelin.

Two different modes of iron uptake are indicated on the basis of these results, as has already been mentioned

(see Discussion, Chapter 1): "soluble" iron being taken up via the mycobactin, while "insoluble" iron and iron from ferriexochelin being assimilated by the exochelin mediated system (see Scheme 2). As already pointed out, it is possible to regard mycobactin as a "store or "overflow" for iron, that is employed in times of feast (to use the definition of Koch (see Introduction)) when the exochelin-mediated iron uptake system has been saturated.

The capability of iron-deficient cells to take up iron supports this hypothesis, not only in the binding of "soluble" iron to the mycobactin as described above, but also in the greater capacity of iron-deficient cells to take up large, non-physiological amounts of iron, compared to iron-sufficient cells (Tables 17 and 18). In this case, presumably all the exochelin produced by the iron-deficient organism will become bound with iron and thereby saturate this iron-uptake system. The extra iron will then be assimilated via the mycobactin "overflow" as previously described (see Discussion, Chapter 1). This mechanism of iron uptake and "storage" has advantages for the organism when viewed from the "feast and famine" standpoint of Koch. By assimilating large amounts of available iron that are in excess to immediate requirements, the organism has gained a supply of a valuable nutrient that will be required during a time of ntrient scarcity or "famine". Besides this advantage of being able to store iron against future requirements, the iron is also held in a tightly bound form that will not be subject to hydroxylation and precipitation. In addition, the iron will

be "leached" into the cell at a slow rate under the action of the non-specific ferrimycobactin reductase; as already described, this rate of iron release will be at a sufficiently slow rate to enable it all to be bound by suitable acceptor molecules and not 'lost' by hydroxylation and precipitation.

Such a mechanism operating *in vitro* is supportable by the above observations. The attainment of a constant intracellular iron concentration by these cells (Tables 17 and 18) may indicate that this mycobactin mediated "overflow" may also be saturable. In addition, on saturation of the exochelin mediated iron-uptake system *in vitro*, iron transport occurs via mycobactin in the manner described here (Stephenson and Ratledge, 1979). The lower capability of iron-sufficient cells to assimilate iron under the same conditions (Tables 17 and 18) presumably reflects the considerably lower levels of mycobactin and exochelin produced by *M. smegmatis* during iron-sufficient growth.

The operation of an 'overflow" mechanism such as this *in vivo* is difficult to substantiate. The *in vivo* environment is almost certainly to be one of "famine", as iron, although plentiful, will usually be in a tightly bound form e.g. ferritin, and thus unavailable to the cell. In this case this effectively iron-deficient environment will stimulate the production of mycobactin and exochelin. Conditions of "feast" occurring, where either iron becomes available in plentiful supply in a soluble form, or the exochelin mediated system is saturated, could presumably occur on rare occasions. Certainly such a mechanism would

indicate the function of mycobactin, whose efficient iron-binding properties would imply a role in iron-transport. This mechanism would also account for the apparent non-involvement of mycobactin in iron transport when using ferriexochelin as a source of iron (Stephenson and Ratledge, 1979).

Chapter 3 Taxonomy Of The *M. avium-intracellulare* Complex

The use of mycobactin as a chemotaxonomic marker has been reviewed by Snow (1970) and Ratledge (1976) as well as in the Introduction to this thesis. Mycobactins have been used in previous taxonomic studies, but only to provide evidence to substantiate proven species boundaries (Snow, 1970). Mycobactins have not before been used as a taxonomic aid to establish whether such a species boundary exists.

Previous evidence has substantially supported the amalgamation of the members of the *M. avium-intracellulare* complex to form a single species, (as reviewed in the Introduction). Evidence presented in this thesis suggests that, although similar, the members of the *M. avium-intracellulare* complex may be separable into two species. Certainly n.m.r. spectroscopy of the mycobactins obtained from the strains examined indicated that the group could be divided into two sub-groups on the basis of the unexplainable peak at 4.0 p.p.m. This was present in all the *M. intracellulare* strains examined, but none of the *M. avium* strains. The substituent tentatively identified as responsible for this anomalous peak was the R_4 moiety on the mycobactin molecule. This may be a proton in *M. avium* strains (as is usually the case in most other species), but is as yet unidentified in *M. intracellulare* strains (Table 29). It is possible that the aliphatic chain characteristic of mycobactins may occur at the R_4 position in the molecule, as seen with mycobactins M and N (Figure 3). However this is unlikely due to the scarcity of this feature amongst the mycobactins;

the aliphatic chain is nearly always found at the R_1 position.

It should be noted that differences in the substituents on the mycobactin molecule are usually apparent during examination using the relatively unrefined technique of t.l.c. Mycobactins S and T, that differ only in the type of alkyl chain present at the R_1 substituent position, are easily separable using this technique. That no differences were seen between the mycobactins from the *M. avium-intracellulare* strains employed when examined by t.l.c. implies that the absorption at 4.0 p.p.m. may be caused by something other than an R group on the mycobactin molecule e.g. just an extra proton. The failure to isolate the fragment of the mycobactin molecule that contained the R_4 group has meant that both the identity of this moiety and the possible cause of the peak at 4.0 p.p.m. are still uncertain. This does not detract however from the fact that n.m.r. spectroscopy does provide a means for separating this complex into two groups.

The majority of evidence presented in this thesis using the mycobactins obtained from members of the *M. avium-intracellulare* complex suggests however that the strains representing *M. avium* and *M. intracellulare* are similar enough to be regarded as one species. The similarity between the mycobactins by t.l.c. (Table 26) and the complete absence of any common features between them using g.l.c. and h.p.l.c. (Tables 27 and 28) would support this proposal. As already stated, the failure to separate between the mycobactins using t.l.c., which in the past has proved capable of separating mycobactins and nocobactins

from different species (Snow, 1970; Ratledge and Patel, 1976) indicates the similarity between the mycobactins. This proposal is supported by the majority of the evidence obtained using other methods of taxonomic analysis (see Introduction). Most workers have shown that the strains representing *M. avium* and *M. intracellulare* are similar enough to be regarded as the same species.

In this case, what is the relevance of the difference in the mycobactins on the basis of the n.m.r. spectroscopy data? The problem arising here with conflicting evidence concerning the 'overlap' of different species may become common as the tools used for taxonomic investigation become more refined. It is likely that observations made using refined techniques such as n.m.r. spectroscopy may reflect differences between organisms that bear no relation to the species structure within the genus. This has been illustrated by Stanford and Grange (1974), who have shown that several strains of one mycobacterial species, *M. fortuitum,* could be regarded as separate species on the basis of certain techniques. They have argued that the bacterial characters the techniques highlight are not concerned with specific variations but with deletional mutations within the species.

The problem has arisen because there is no clear definition of how different two organisms have to be in order to constitute two species. It is therefore likely that the strains of *M. avium* and *M. intracellulare* examined here are similar enough as to be regarded as belonging to the same species. The differences observed

between the strains that have caused confusion both here and amongst other taxonomists are not significant enough to warrant dividing the complex into two species. This is reiterated by Wayne (1978) who stated that the differences observed between strains of this complex bore no resemblance to any species differences. Stanford has also supported this (J. L. Stanford, personal communication) when suggesting that the differences reported by other members of the collaborative group funded by this current grant from The Welcome Trust, may not be significant in regarding this complex as containing two species.

Appendix I

The n.m.r. spectra of mycobactins P, S, the mycobactins obtained from the *M. avium-intracellulare* strains examined and the cobactin fragments from mycobactins Av2 and Av34 are included here, rather than the main body of the text.

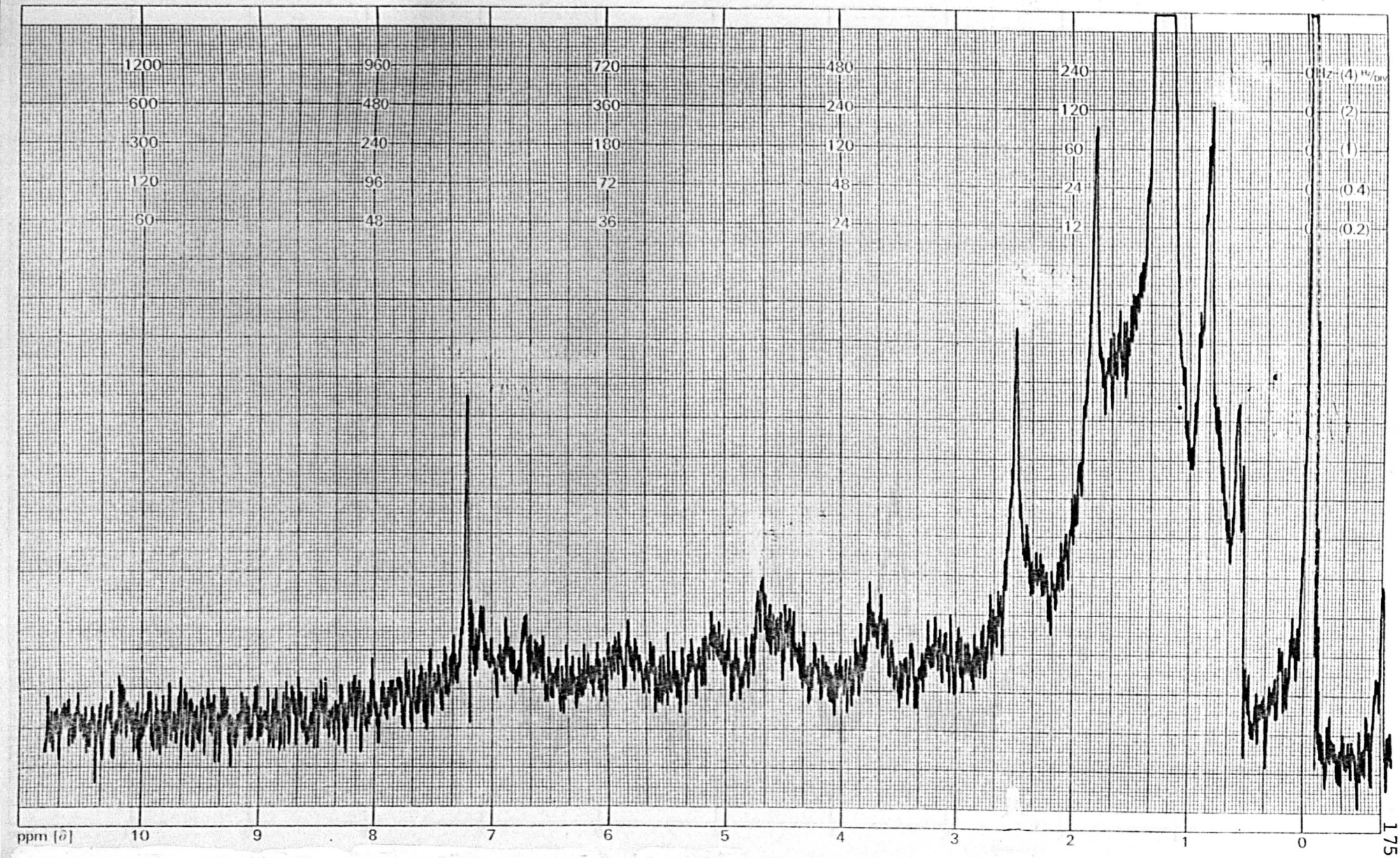


FIGURE 11 N.m.r Spectrum of Mycobactin P

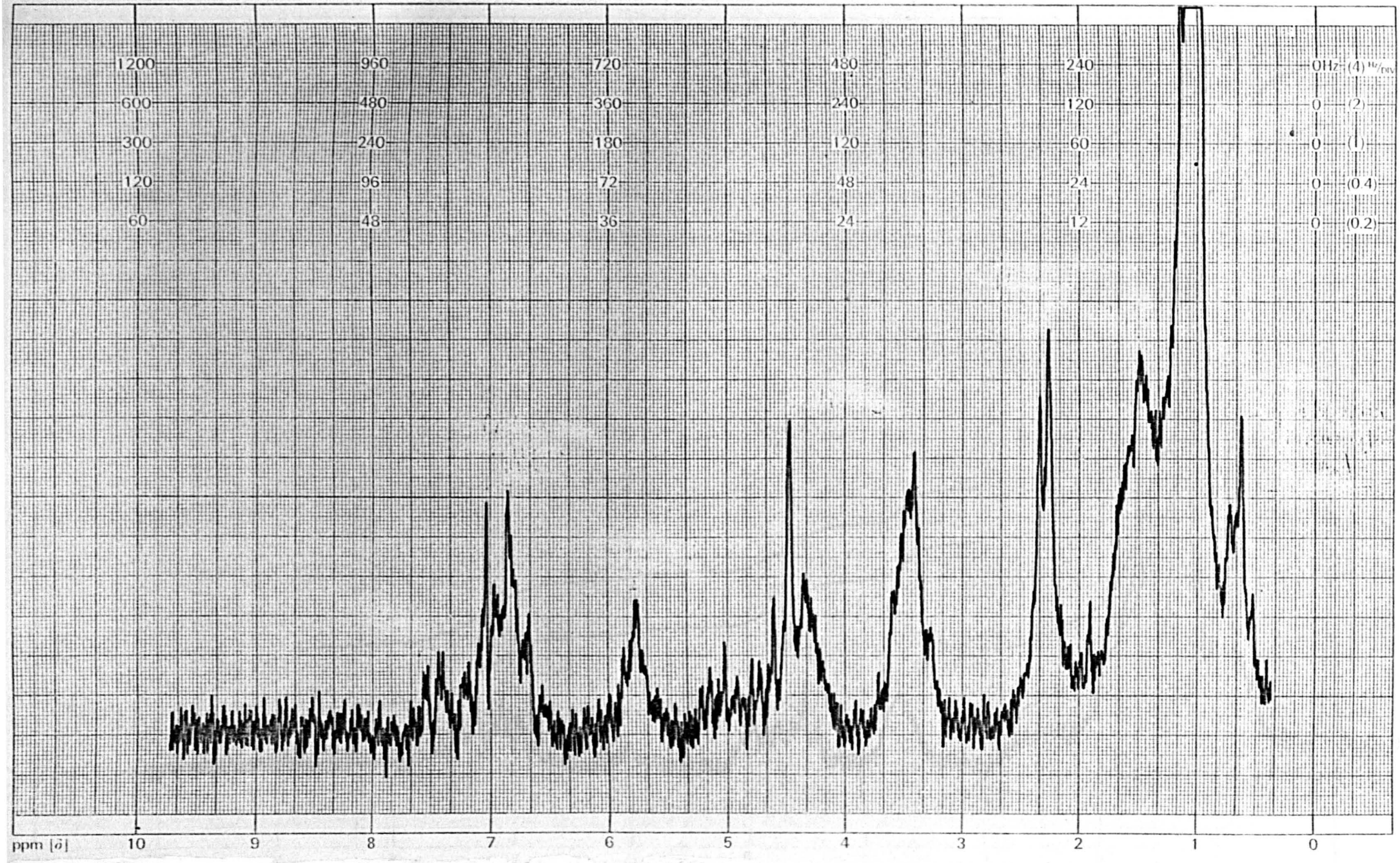


FIGURE 12 N.m.r. Spectrum of Mycobactin S

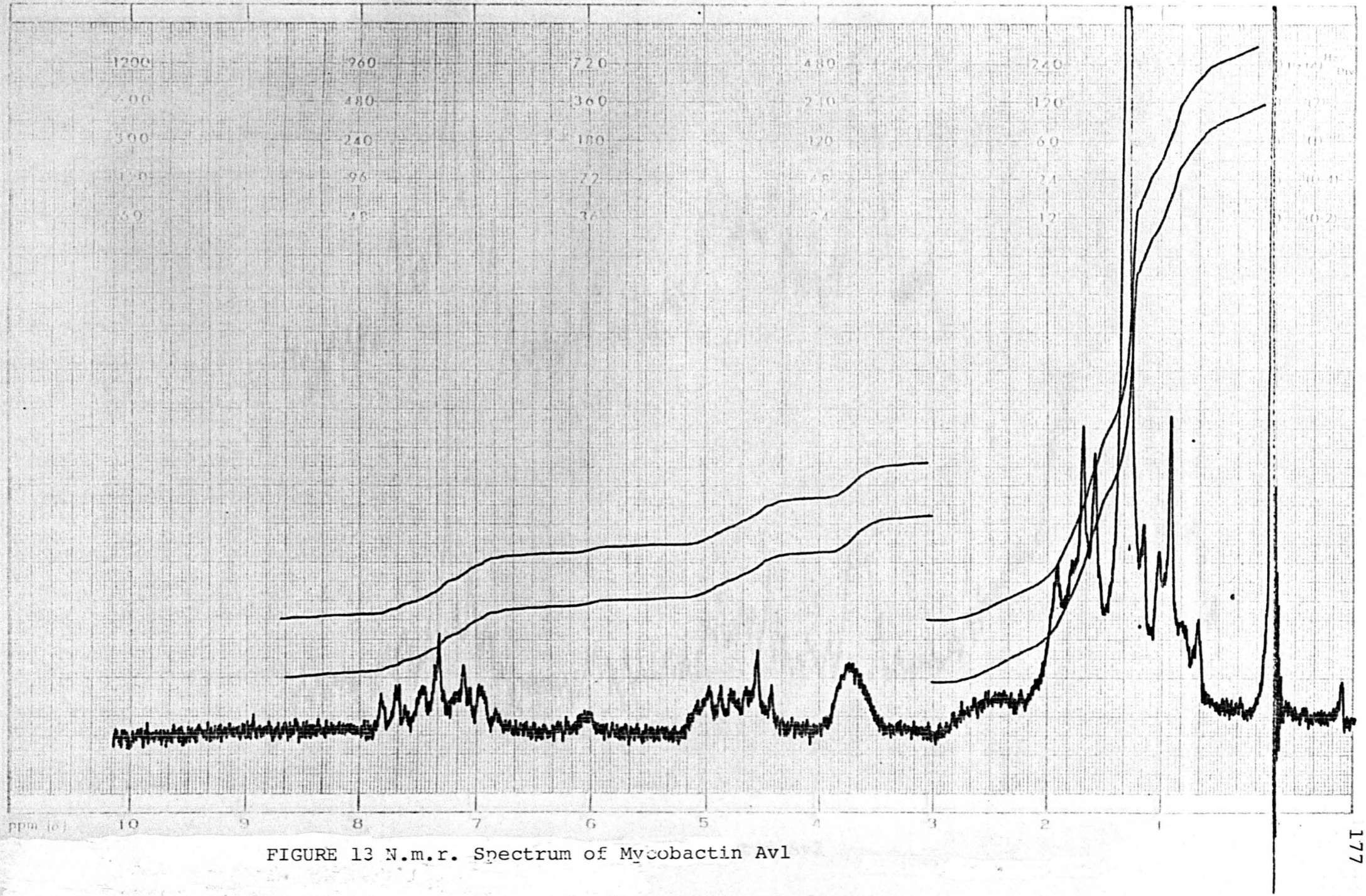


FIGURE 13 N.m.r. Spectrum of Mycobactin Av1

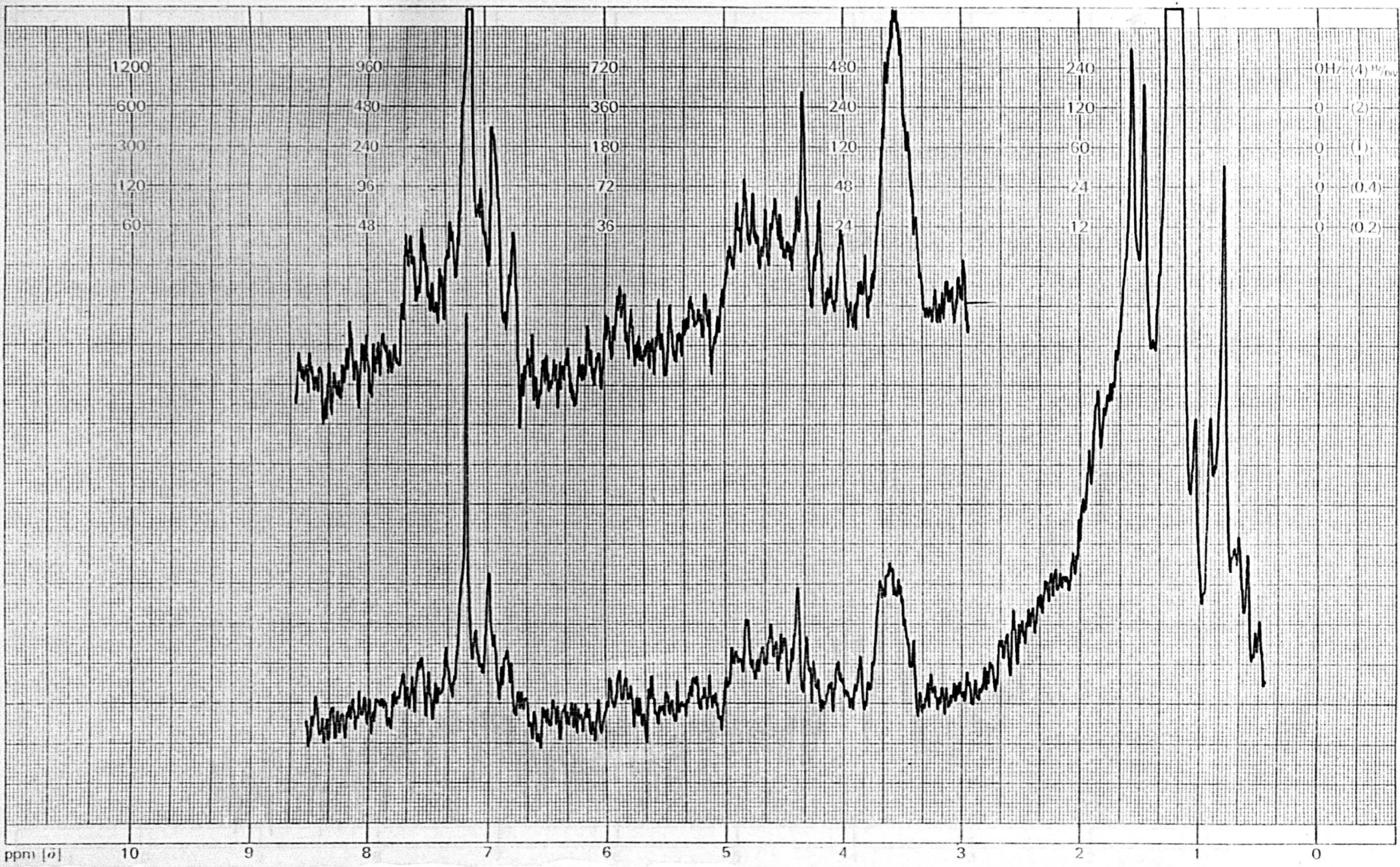


FIGURE 14 N.m.r. Spectrum of Mycobactin Av2

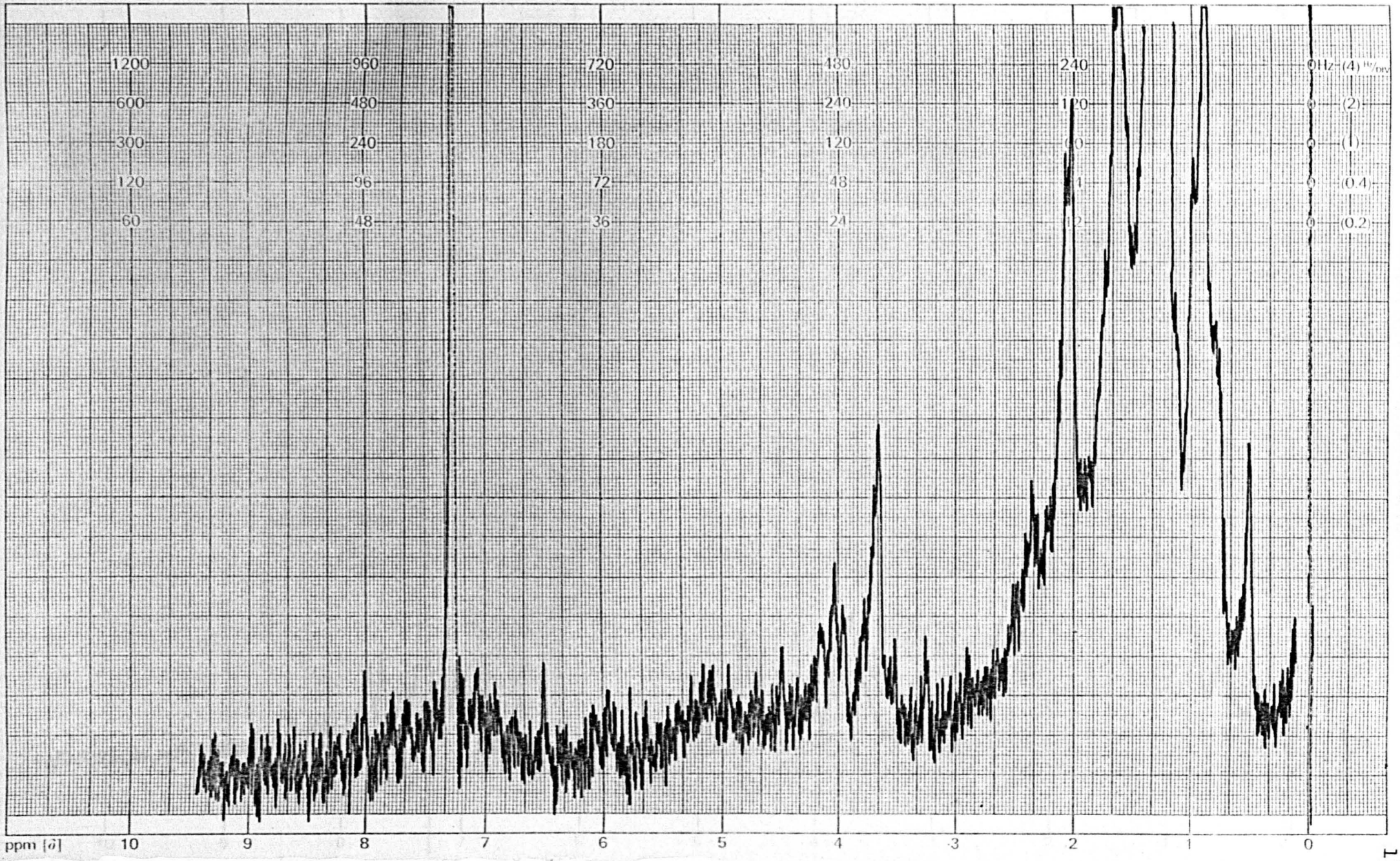


FIGURE 15 N.m.r. Spectrum of Mycobactin Av4

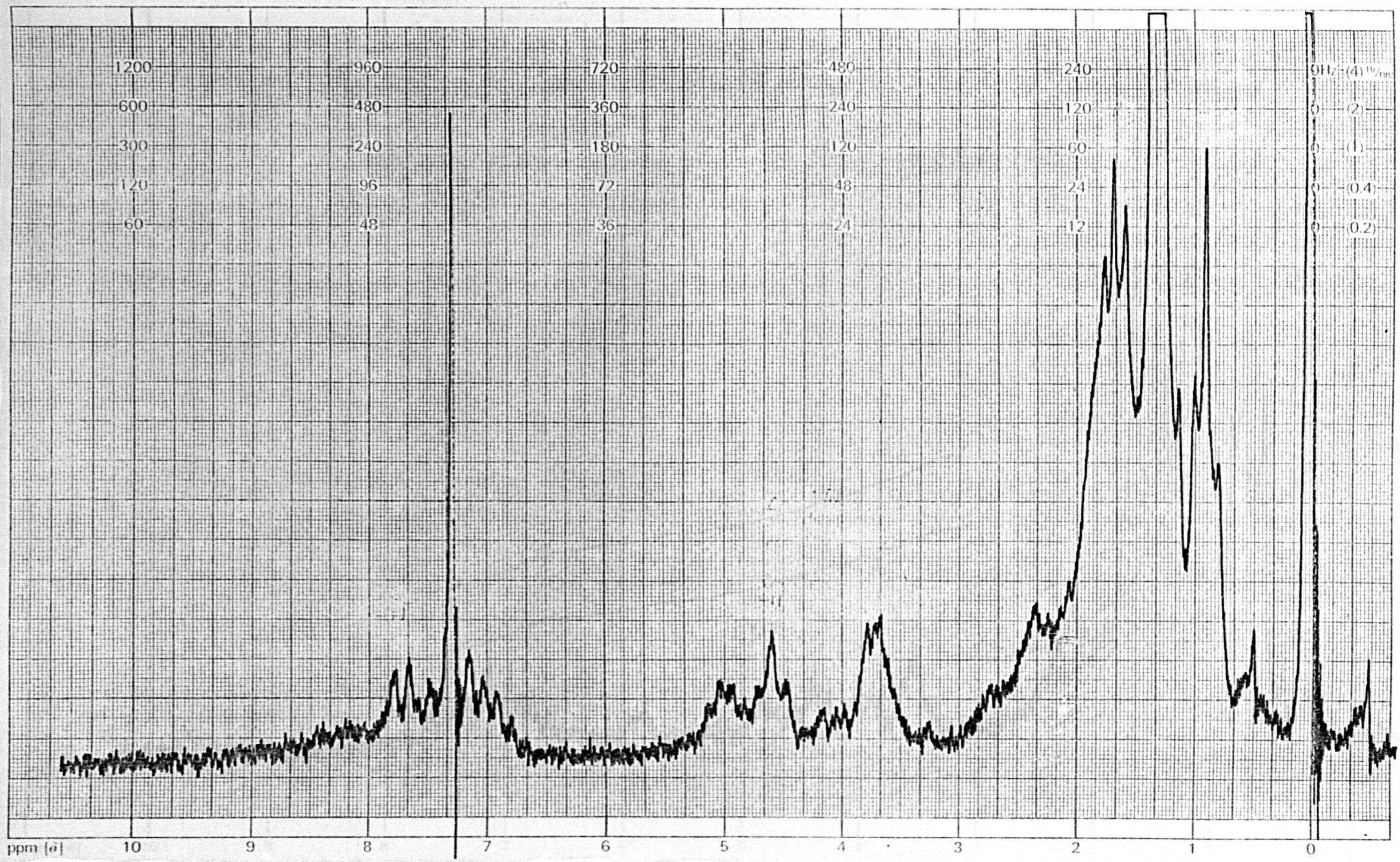


FIGURE 16 N.M.r. Spectrum of Mycobacton Av8

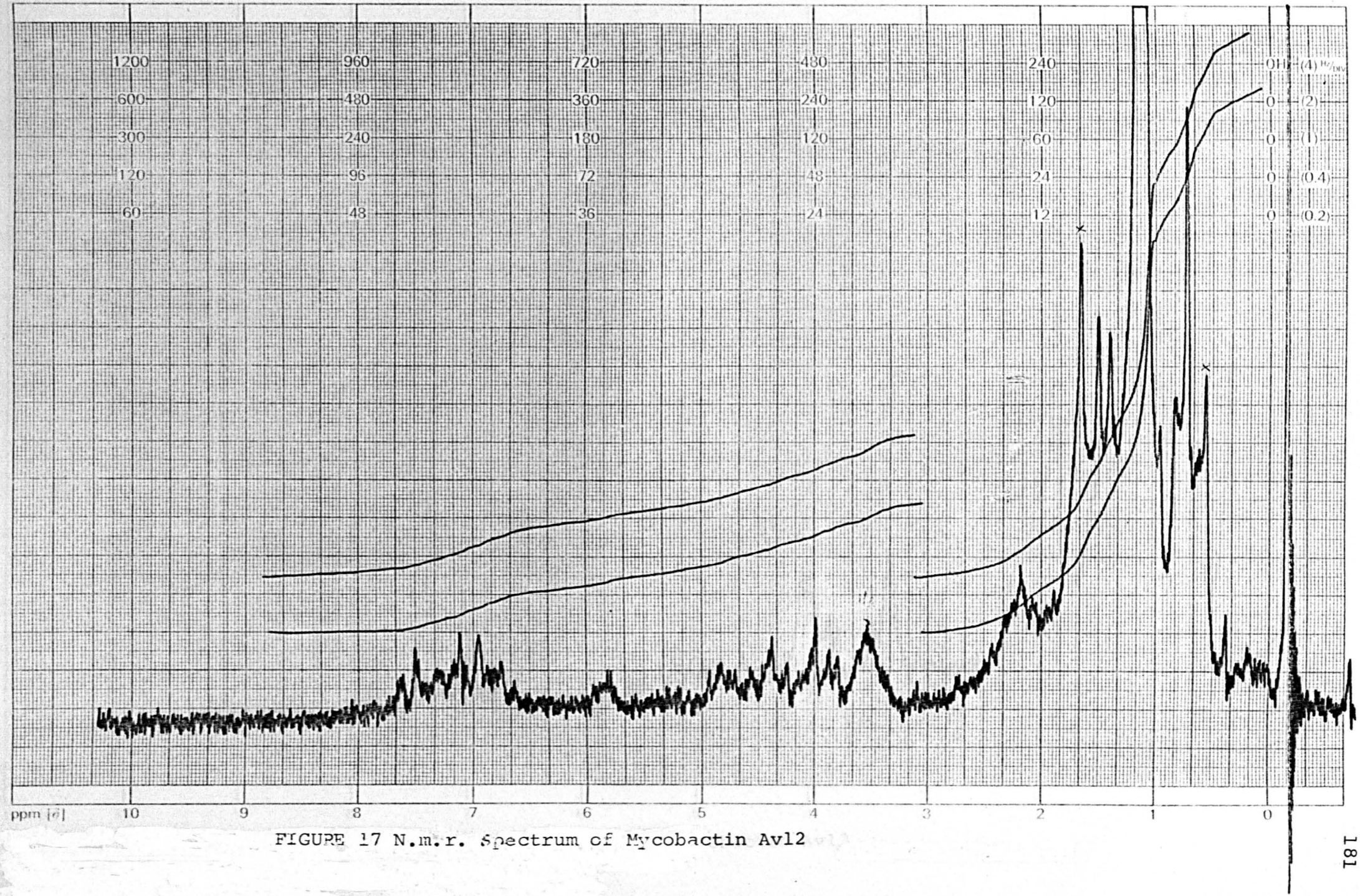


FIGURE 17 N.m.r. Spectrum of Mycobactin Av12

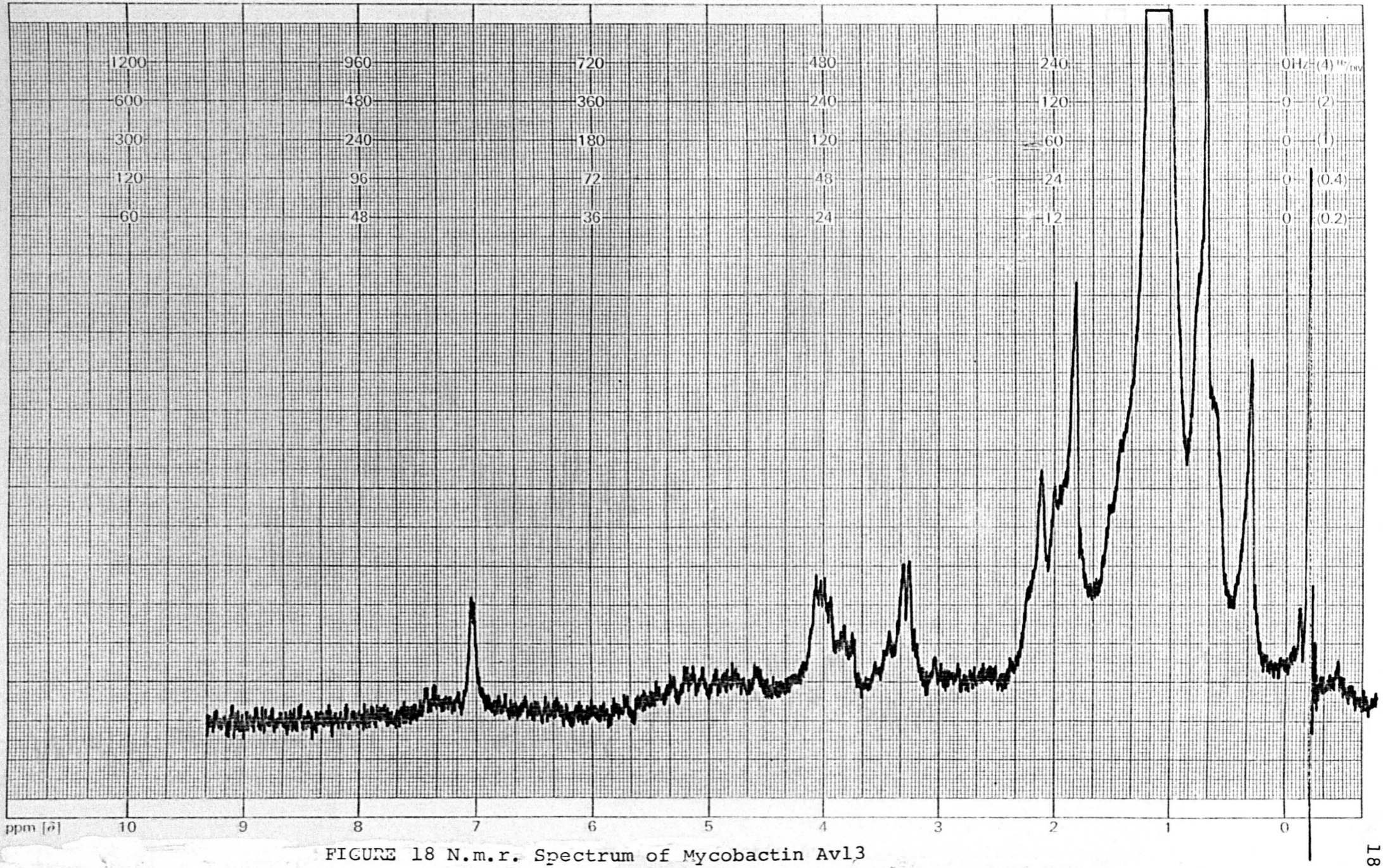


FIGURE 18 N.m.r. Spectrum of Mycobactin Av13

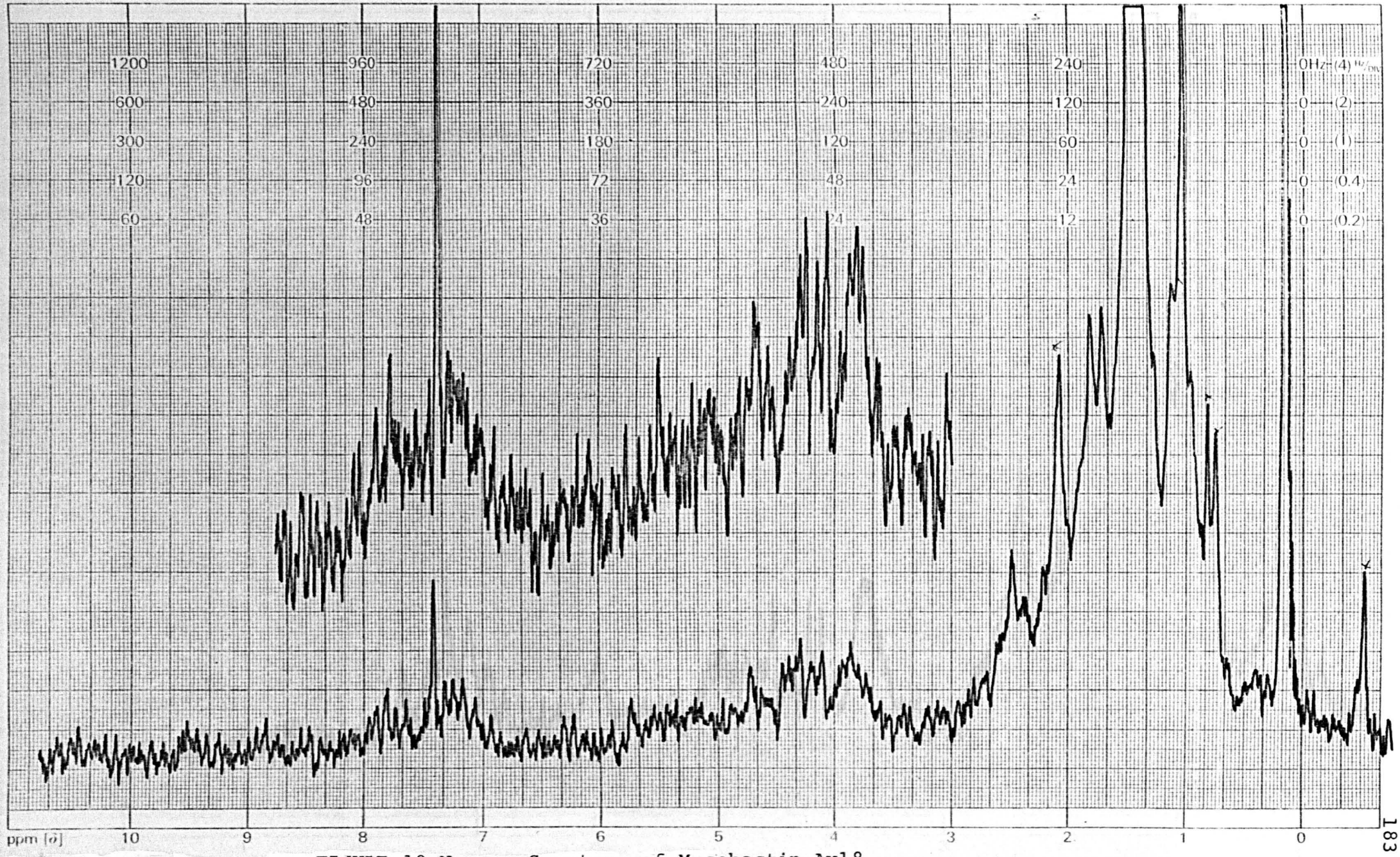


FIGURE 19 N.m.r. Spectrum of Mycobactin Av18

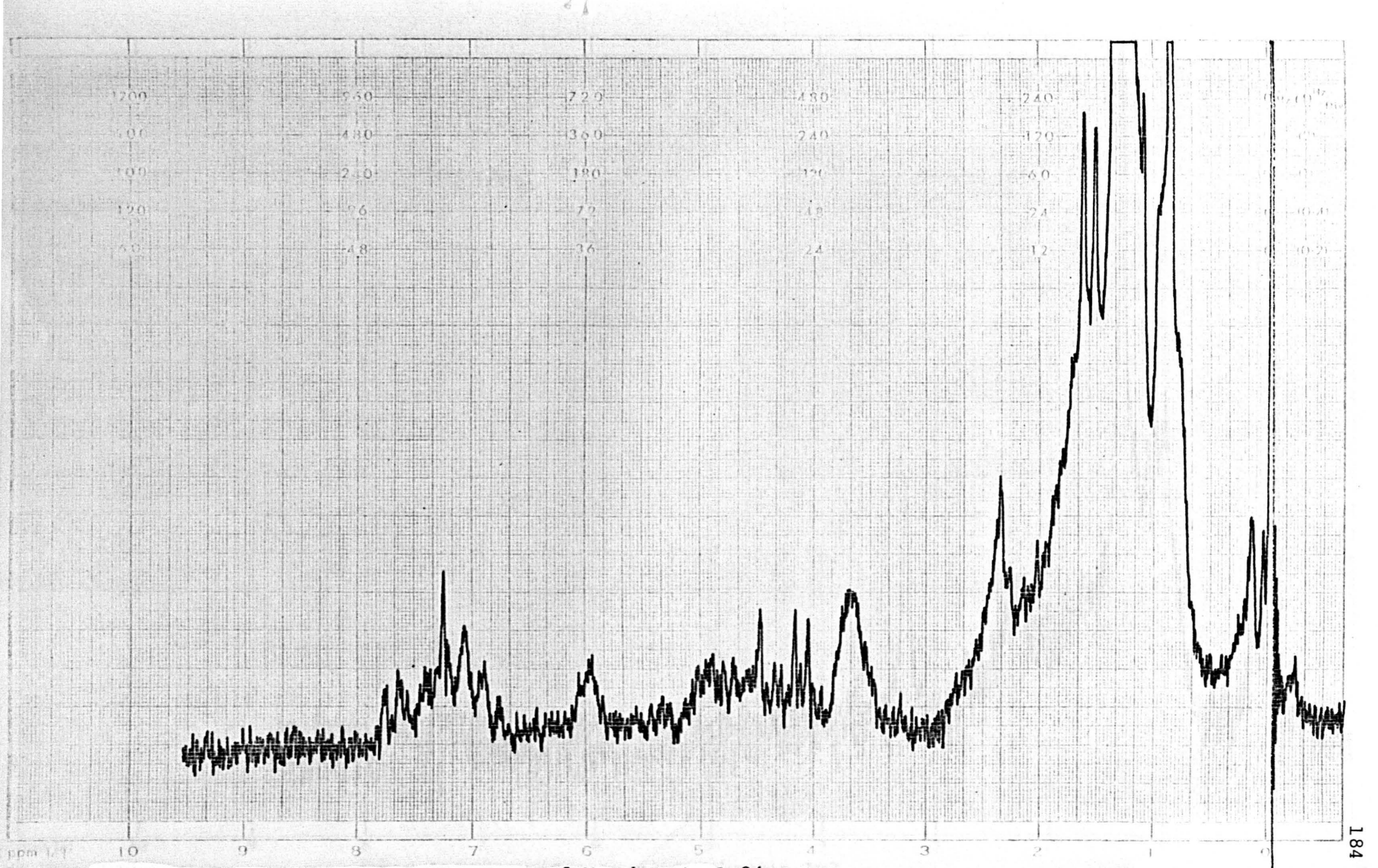


FIGURE 20 N.m.r. Spectrum of Mycobactin Av34

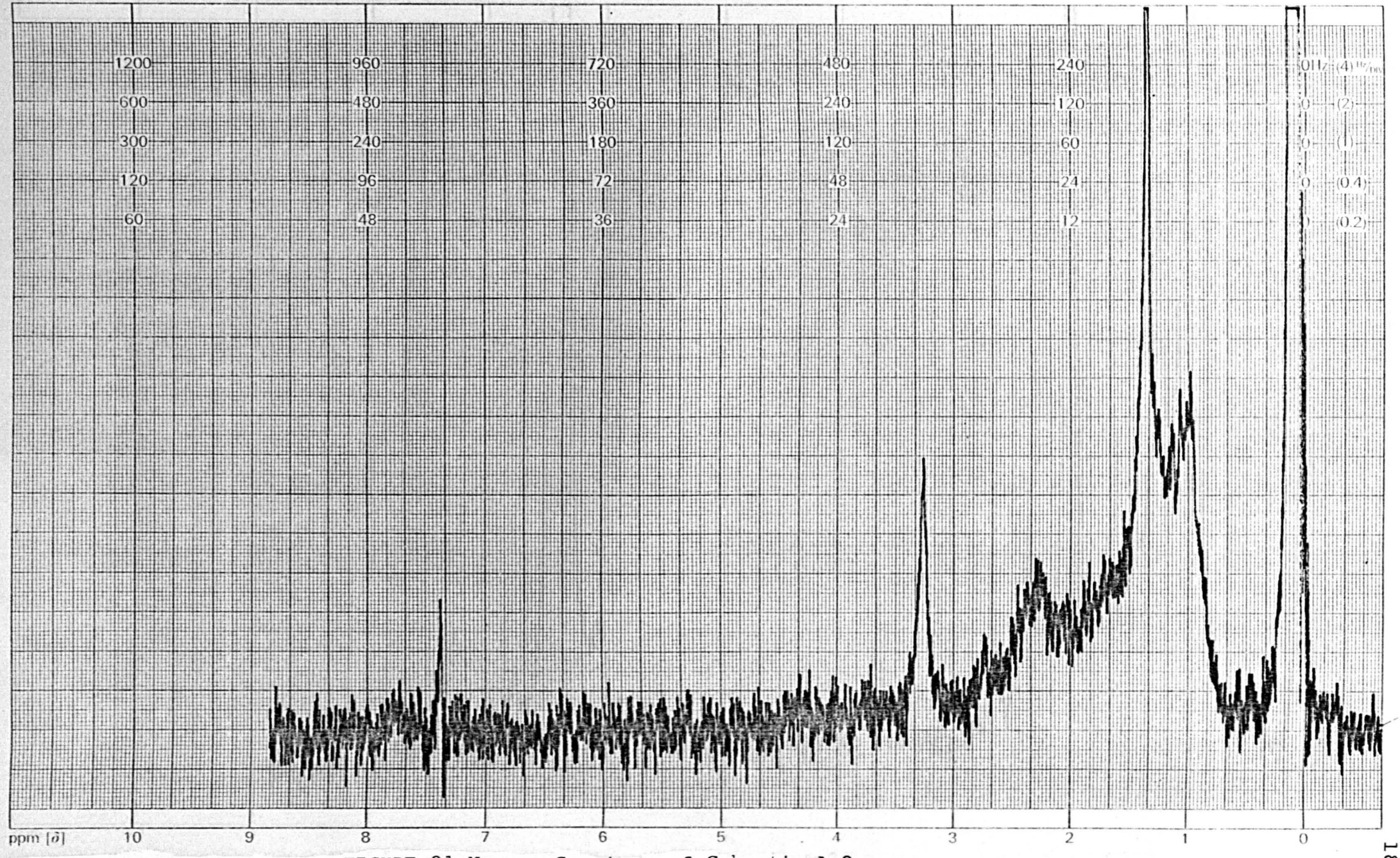


FIGURE 21 N.m.r. Spectrum of Cobactin Av2

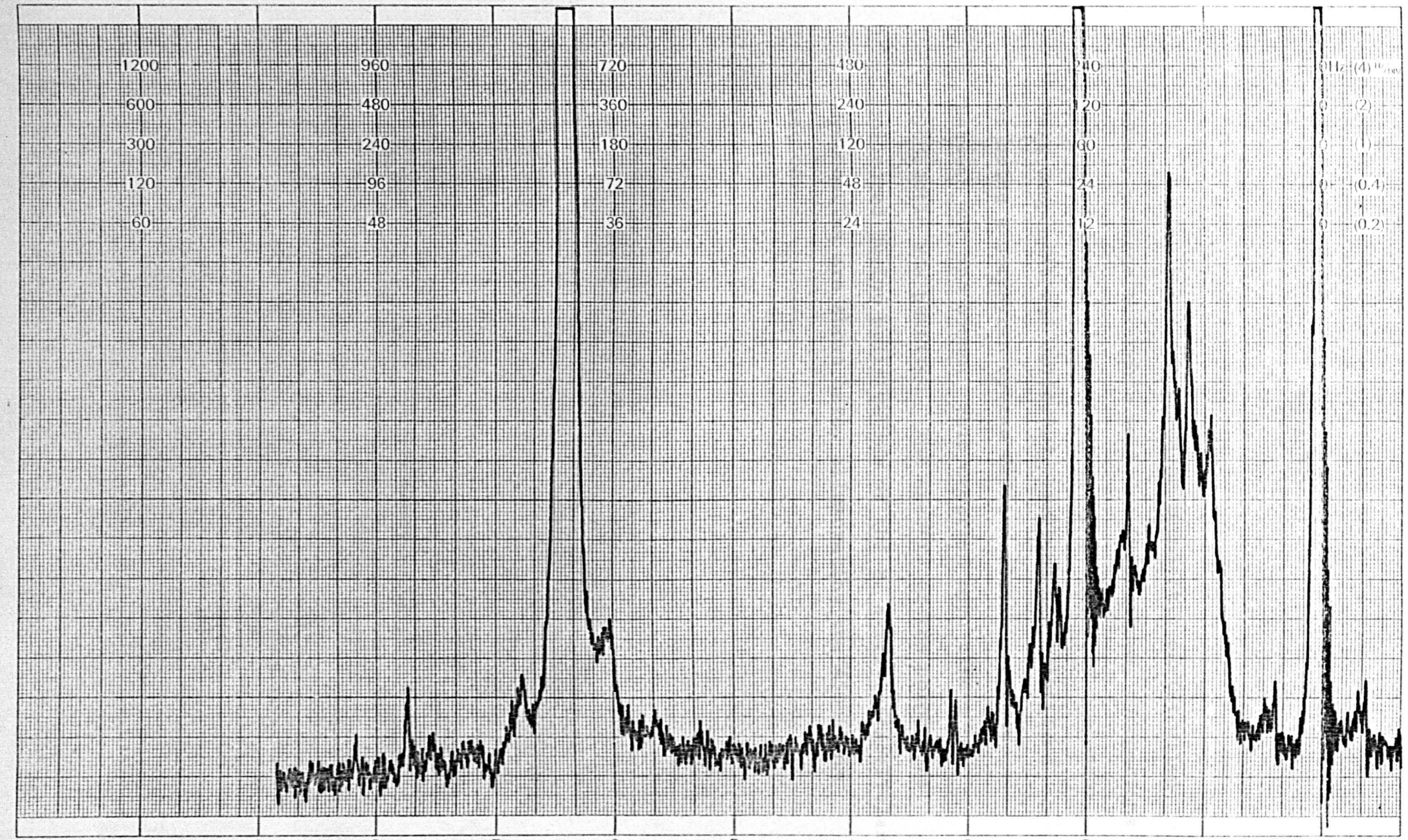


FIGURE 22 N.m.r. Spectrum of Cobactin Av34

Appendix II

During the final stage of mycobactin purification, namely passage through a sephadex LH20 column, mycobactins separated from a slower-running, pink-coloured component. This component was collected, examined and identified as being a porphyrin by the characteristic spectrum between 450 and 650nm. This occurred each time a mycobactin was purified. The spectra of the porphyrins collected during purification of the *M. avium-intracellulare* mycobactins are shown here, illustrating the 450-650nm region.

Figure 23. Spectrum Of Porphyrin Obtained During Purification of Mycobactin Av1

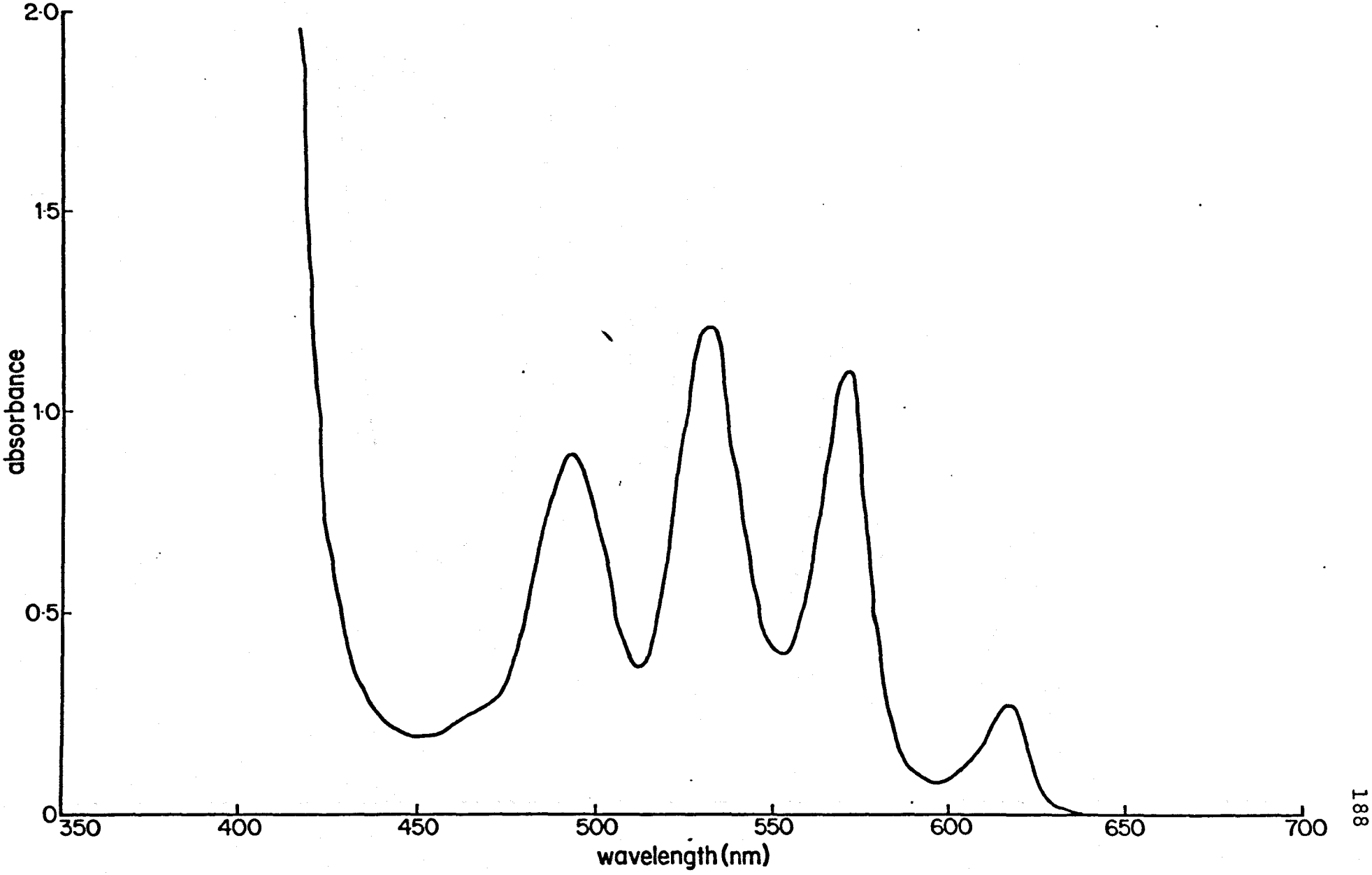


Figure 24. Spectrum Of Porphyrin Obtained During Purification of Mycobactin Av2.

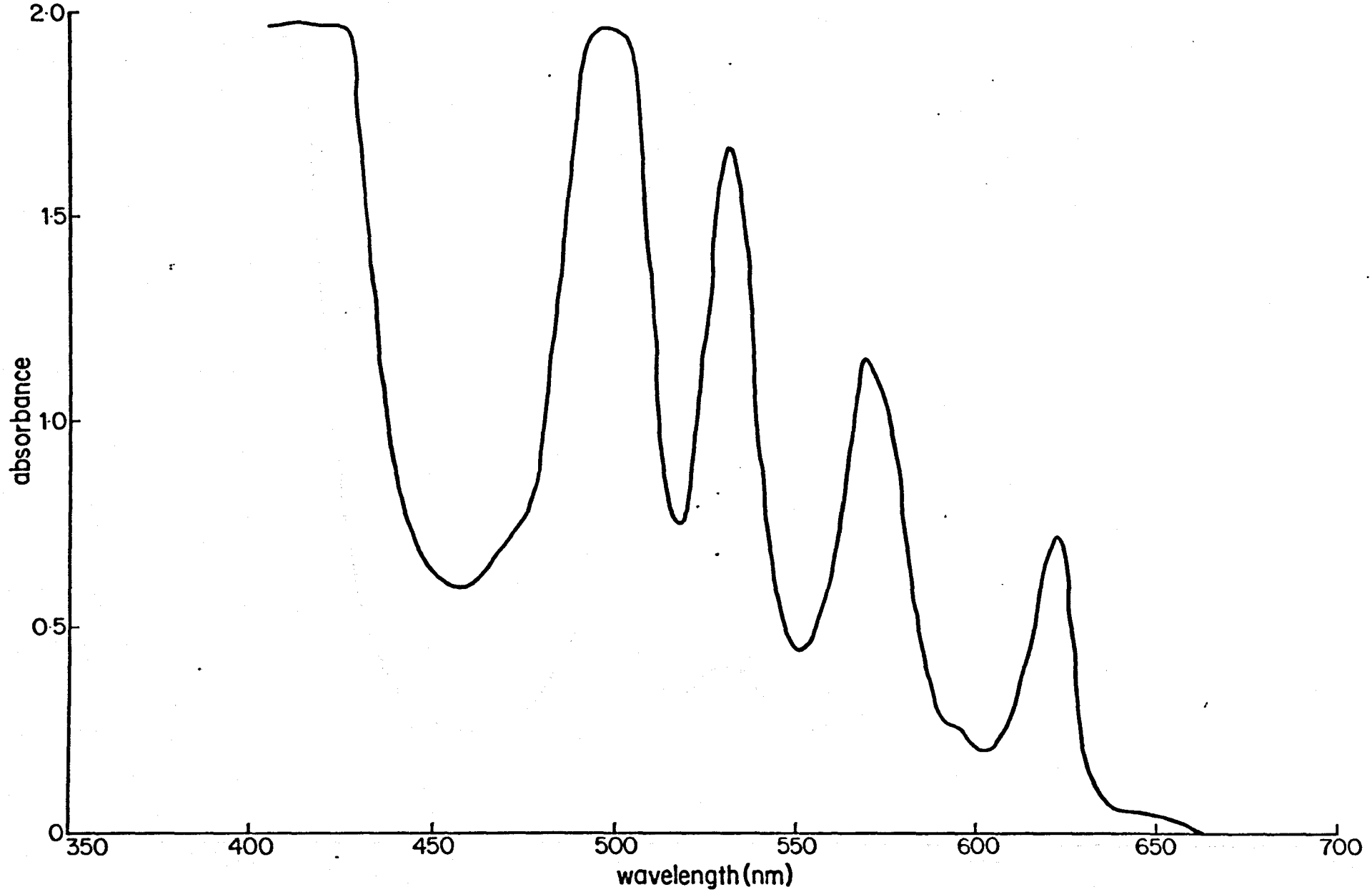


Figure 25. Spectrum Of Porphyrin Obtained During Purification of Mycobactin Av4.

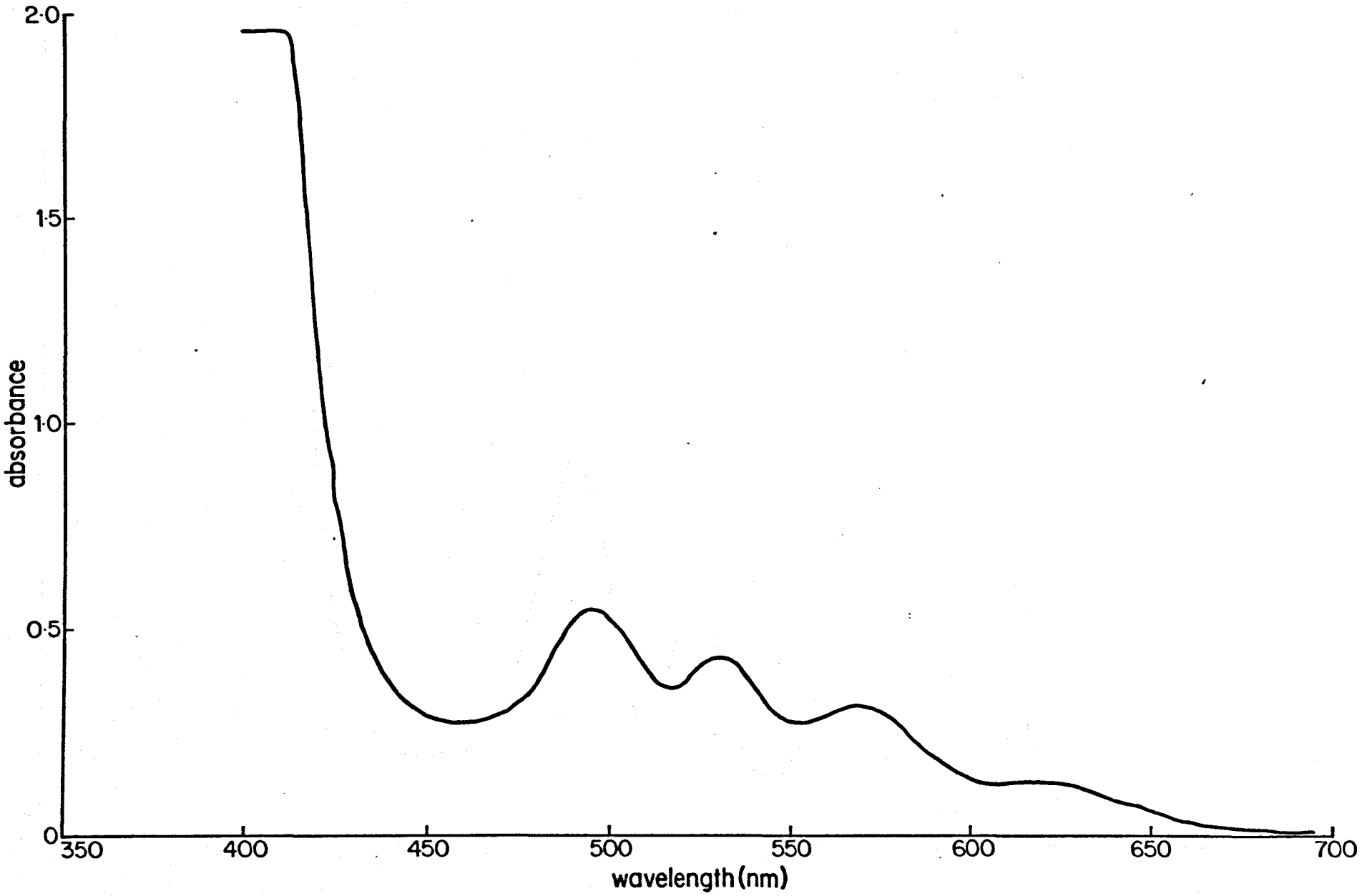


Figure 26. Spectrum Of Porphyrin Obtained During Purification of Mycobactin Av8.

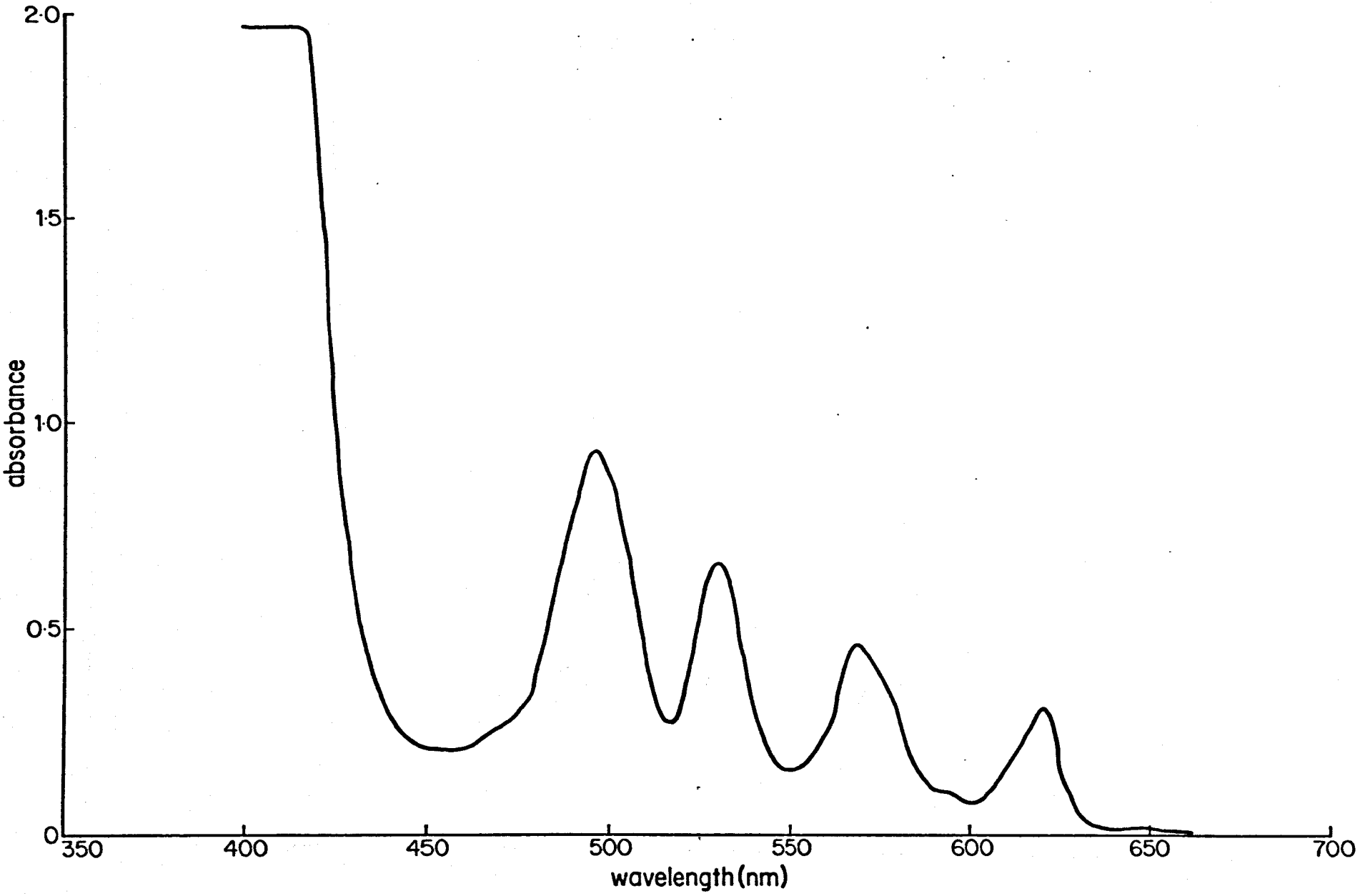


Figure 27. Spectrum Of Porphyrin Obtained During Purification Of Mycobactin Av12.

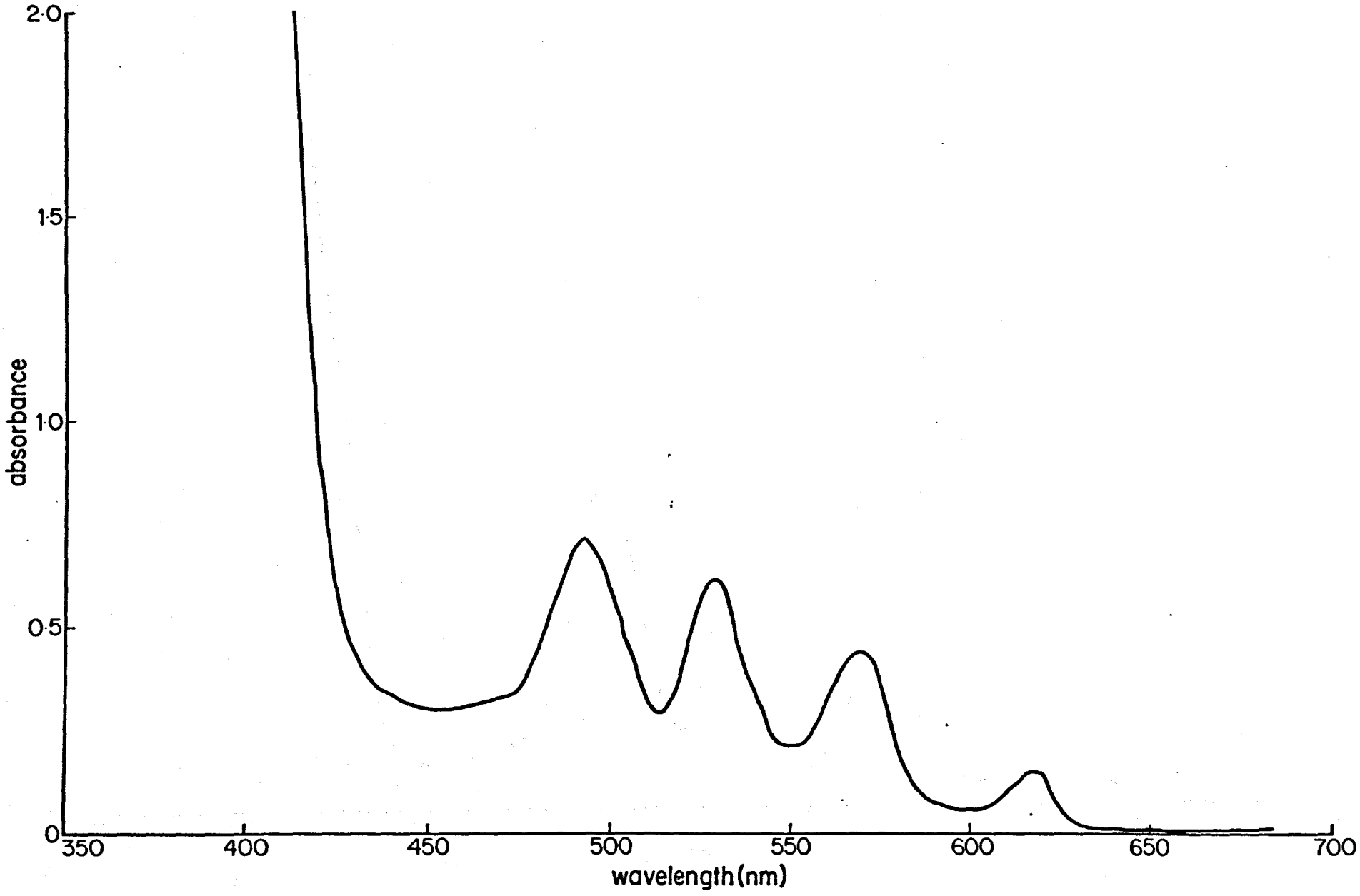


Figure 28. Spectrum Of Porphyrin Obtained During Purification Of Mycobactin Av18.

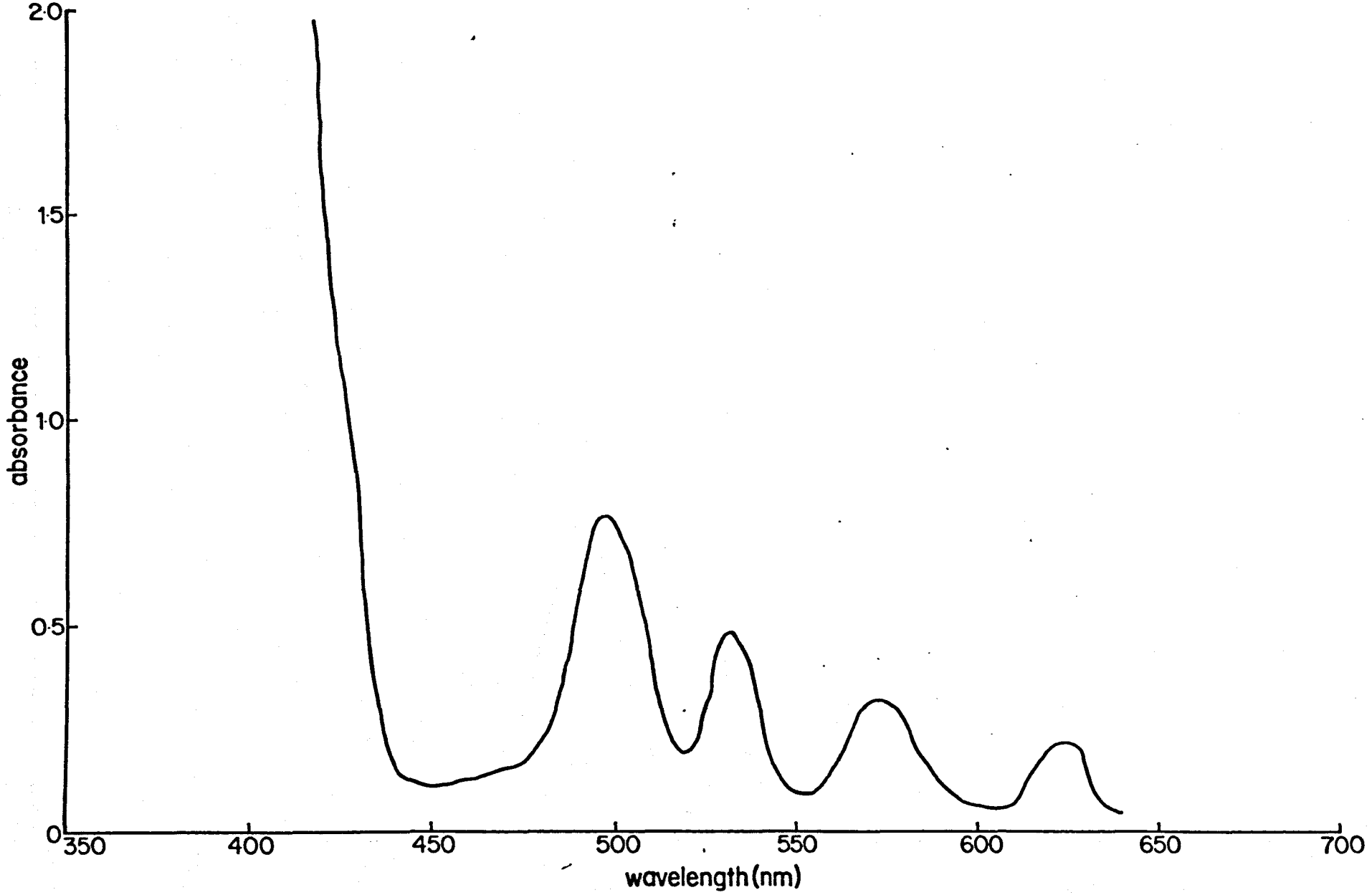
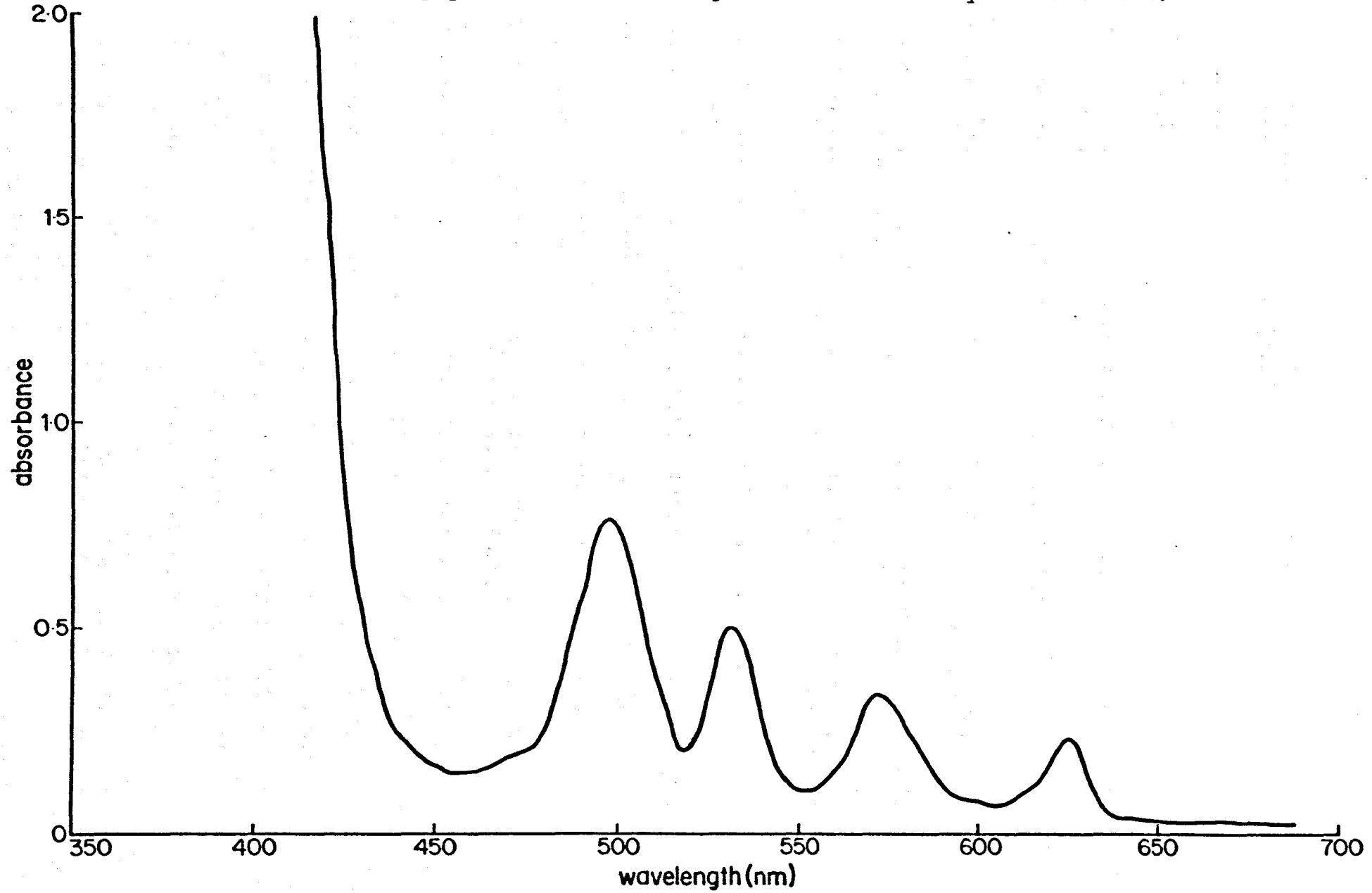


Figure 29. Spectrum Of Porphyrin Obtained During Purification Of Mycobactin Av34.



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